Cytoprotective Properties of Erythropoietin in Nonerythropoietic Tissues: From Erythrocytes to the Heart

---

Dissertation

zur

Erlangung der naturwissenschaftlichen Doktorwürde

(Dr. sc. nat.)

vorgelegt der

Mathematisch-naturwissenschaftlichen Fakultät

der

Universität Zürich

von

Deyan Nikolaev Mihov

aus Bulgaria

Promotionskomitee

Prof. Dr. Max Gassmann (Vorsitz)

Dr. Anna Yu. Bogdanova (Leitung der Dissertation)

Prof. Dr. Christian Grimm

Dr. Hans U. Lutz

Zürich, 2009

TABLE OF CONTENTS

1. ABREVIATIONS

^{125}I-Epo	Radioactively labeled, iodinated erythropoietin
Akt	Protein kinase B
BFU-E	Burst-Forming Units-Erythroid
CFU-E	Colony-Forming Units-Erythroid
eNOS	Endothelial type (type 3) nitric oxide synthase
Epo	Erythropoietin
EpoR	Erythropoietin Receptor
GSH	reduced Glutathione
GSK-3β	Glycogen synthase kinase 3β
GSSG	oxidized Glutathione
HIF-1	Hypoxia Inducible Factor 1
PI3K	Phosphatidylinositol-3-kinase
I-R	Ischemia-Reperfusion
Jak2	Janus kinase 2
KCC	K^+-Cl^--cotransporter
L-Arg	L-arginine
L-Cytr	L- citrulline
L-NAME	N^G-Nitro-L-Arginine Methyl Ester
L-NMA	N^G-Monomethyl-L-arginine
MAPK	Mitogen-Activated Protein Kinase
NKCC	Na^+-K^+-Cl^--cotransporter
NO	Nitric Oxide
NOS	Nitric Oxide Synthase
PLC-1γ	Phospholipase C-1γ
RBCs	Red Blood Cells
rhEpo	recombinant, human Erythropoietin
STAT-5	Signal Transducer and Activator of Transcription 5

2. SUMMARY

Erythropoietin (Epo) is the major regulator of red blood cell production. At present human recombinant Epo (rhEpo) is widely used in clinics to treat anemia. Apart from this, Epo is considered as a pleiotropic, cytoprotective factor with impressive therapeutic potential in treatment of stroke and myocardial infarction. Increasing number of reports describes Epo-induced responses in a number of non-hematopoietic tissues and cell types, including neurons and cardiomyocytes. However the primary cellular targets of rhEpo and the signaling cascades it activates *in vivo* remain unclear.

The aim of the present study was to investigate the non-hematopoietic properties of rhEpo and to characterize the primary cellular targets and downstream signaling pathways activated by the cytokine in mammalian erythrocytes and heart tissue. When rhEpo is injected intravenously, the first cells that face the cytokine are the blood cells and the vascular endothelium. Therefore, our work focused on rhEpo-induced responses in mature, mammalian red blood cells (RBCs) on one hand. On the other, we investigated the putative cardioprotective properties of rhEpo against ischemia-reperfusion injury (I-R) in the myocardium, using *in vivo,* heterotopic rat heart transplantation model and isolated cardiomyocytes.

We found that mouse erythrocytes possess specific Epo-biding sites, bearing characteristics of “classical” EpoR. Reticulocytes and young erythrocytes have a markedly higher number of Epo-binding sites in comparison to adult and senescent RBCs, suggesting that the sensitivity and responsiveness to the cytokine will decrease with erythrocyte age. Treatment of total mouse RBC population with rhEpo triggered a range of primary and secondary effects including activation of the endothelial type nitric oxide synthase, alteration in the cellular half-cell redox potential and changes in ion and glucose transport. Nitric oxide, redox state, ion/water balance and glucose metabolism are determinants of erythrocyte rheology, life span and oxygen carrying capacity. Taken together our findings imply that along with the increase in the red cell number, rhEpo causes multiple acute responses in the already existing RBCs that improve tissue oxygenation under hypoxic conditions and protects erythrocytes from oxidative stress and premature senescence.

Cytoprotective effects of rhEpo were also observed in hearts exposed to cold global ischemia-reperfusion (I-R) injury *in vivo*. The data we obtained, revealed that rhEpo is a potent anti-necrotic factor that reduces tissue oedema, and mechanical and oxidative stress triggered by I-R. We showed for the first time that, when administered intravenously (iv), rhEpo does not pass through the blood vessel wall and does not interact directly with the cardiomyocytes. The primary *in vivo* cellular targets of the cytokine are the vascular endothelial cells. The observed cardioprotective properties of rhEpo are most probably mediated by endothelium-derived paracrine factors, one of which is nitric oxide.

In addition the *in vitro* experiments we performed proved that neonatal cardiomyocytes can bind and respond directly to rhEpo treatment. In both coronary endothelial cells and cardiomyocytes, cytokine administration results in activation of the PI3K/Akt pathway and an elevation of the NO production. In the cardiomyocytes these effects trigger secondary changes in the activity of Na^+/K^+-ATPase. These direct Epo-induced effects on cardiac cells could be important for the normal heart development.

3. ZUSAMMENFASSUNG

Erythropoietin (Epo) ist der Hauptregulator der Produktion der roten Blutkörperchen. Heutzutage wird menschliches, rekombinantes Epo (rhEpo) oft in der Klinik verwendet um Anämien zu behandeln. Im Weiteren ist Epo ein pleiotroper zytoprotektiver Faktor mit einem beträchtlichen therapeutischen Potential für die Behandlung von Hirnschlägen und Herzinfarkten. Eine zunehmende Anzahl Berichte beschreibt Reaktionen auf rhEpo von verschiedenen nicht-blutbildenden Zelltypen und Geweben wie zum Beispiel Neuronen und Kardiomyozyten. Die primären zellulären Ziele und Signalwege, die *in vivo* durch dieses Zytokin aktiviert werden, sind jedoch unklar.

Das Ziel dieser Studie war es, die nicht-blutbildenden Eigenschaften von hrEpo zu erforschen und die primären zellulären Ziele und Signalwege zu charakterisieren, die durch dieses Zytokin in von Säugetieren stammenden Erythrozyten und Herzgeweben aktiviert werden. Wird rhEpo intravenös injiziert, gelangen die roten Blutkörperchen und das vaskuläre Endothel zuerst mit dem Zytokin in Kontakt. Deshalb fokussiert unsere Arbeit auf der Erforschung der Reaktion von reifen roten Blutkörperchen auf rhEpo und dessen kardioprotektiven Eigenschaften im Fall von Ischämie-Reperfusions-Vorfällen. Dazu verwendeten wir ein heterotopisches *in vivo* Rattenherztransplantations-Model und isolierte Kardiomyozyten.

Wir zeigten, dass Erythrozyten von Mäusen spezifische Epo-Bindungsstellen besitzen, ähnlich den klassischen Epo-Rezeptoren. Retikulozyten und junge Erythrozyten weisen bedeutend mehr Epo-Bindungsstellen auf als reifen und alternde rote Blutkörperchen, was darauf hindeutet, dass die Sensitivität und Reaktionsfähigkeit auf Epo mit dem Zell Alter abnimmt. Die Behandlung von roten Blutkörperchen mit rhEpo hat verschiedene primäre und sekundäre Auswirkungen auf die Aktivierung der endothelialen Stickoxydsynthetase, auf Veränderung des zellulären Halb-Zell-Redoxpotentials und des Ionen- und Glukosetransports. Stickoxid, Redoxzustand, das Ionen/Wasser-Gleichgewicht und der Glukosemetabolismus bestimmen die Rheologie, Lebensdauer und Sauerstoffladekapazität der roten Blutkörperchen. Zusammenfassend deuten unsere Resultate darauf hin, dass Epo nicht nur zu einer Zunahme der Anzahl roten Blutkörperchen führt, sondern durch seine Wirkung auf bereits vorhandene

Erythrozyten die Sauerstoffversorgung hypoxischer Gewebe verbessert und die roten Blutkörperchen vor oxidativem Stress und frühzeitiger Alterung schützt.

Zytoprotektive Wirkungen von Epo wurden auch *in vivo* beobachtet; in Herzen mit kaltem globalem Ischämie-Reperfusions-Vorfall. Unsere Daten zeigen, dass rhEpo eine potente Wirkung gegen Nekrose zeigt und die Gewebsödeme sowie den mechanischen und oxidativen Stress verhindert, die durch den Vorfall hervorgerufen werden. Wir zeigten zum ersten Mal, dass intravenös verabreichtes rhEpo die Gefässwände nicht durchs und so nicht direkt mit den Kardiomyozyten in Kontakt tritt. *In vivo* sind demzufolge die Endothelzellen die primären zellulären Ziele des Zytokins. Die kardioprotektiven Eigenschaften von rhEpo beruhen daher höchstwahrscheinlich auf vom Endothel stammende parakrine Faktoren wie zum Beispiel Stickoxid.

Im Weiteren bewiesen unsere *in vitro*-Experimente, dass neonatale Kardiomyozyten rhEpo direkt binden können. Sowohl koronale Endothelzellen als auch Kardiomyozyten aktivieren die PI3K/Akt Signalwege und steigern die Stickoxid-Produktion, wenn sie mit rhEpo behandelt werden. In den Kardiomyozyten führt dies zu veränderter Aktivität der Na^+/K^+-ATPase. Diese direkten, von Epo hervorgerufenen Veränderungen der Herzzellen könnten während der normalen Entwicklung des Herzens eine wichtige Rolle spielen.

4. INTRODUCTION

4.1 Erythropoietin

More than a century ago Carnot and Deflandre postulated the existence of a humoral blood-born factor (hemopoietine) controlling the production of red blood cells (Fisher, 2003). The following decades of research revealed that erythropoietin (Epo) is the major regulator of survival, proliferation and differentiation of the erythroid progenitors in the bone marrow. Since 1977 Epo is used routinely in clinics to treat anemia (Fisher, 2003; Ng *et al.*, 2003). Recent findings indicate that Epo is a pleiotropic, cytoprotective factor with impressive therapeutic potential in treatment of different neurodegenerative and cardio-vascular disorders (Sasaki, 2003; Ghezzi *et al.*, 2004; Jelkmann, 2007; Arcasoy, 2008; Maiese *et al.*, 2008).

4.1.1 Structure of erythropoietin

The human Epo gene is localized in the middle of the long arm (q11-q22) of chromosome 7 (Powell *et al.*, 1986). The gene exists as a single copy and is composed of five exons and four introns (Koury *et al.*, 1992). Epo genes of monkey (Lin *et al.*, 1986) and mouse (McDonald *et al.*, 1986), show 92% and 80% homology with the human gene, respectively. The human Epo gene encodes a single polypeptide, a 193-amino acid prohormone. The amino acid leader sequence of 27 residues is cleaved prior to secretion (Lin *et al.*, 1985). In addition, the carboxy-terminal arginyl residue is lost during the posttranslational modification and the active form of the hormone, comprising 165 amino acids, is released into circulation.

Epo is highly glycosylated with approximately half of its molecular weight derived from carbohydrates (Maiese *et al.*, 2005; Maiese *et al.*, 2008). Epo contains four glycosylated chains shown in Figure 1A including three N-linked and one O-linked, acidic oligosaccharide side chains (Fisher, 1997). N-linked glycosylation sites are located in the positions 24, 38, and 83 of aspartyl residues, while the O-linked glycosylation site is at Serine-126 (Fig. 1A, empty circles; Fig 1B, red arrows). In addition, the structure of Epo is maintained by the presence of two disulfide bonds formed between cysteines at position 7 and 160 and at positions 29 and 33 (Fig. 1A) (Fisher, 1997). The production, secretion, stability

and biological function of the hormone depends on the integrity of the oligosaccharide chains and disulfide bonds (Li *et al.*, 2004; Maiese *et al.*, 2008).

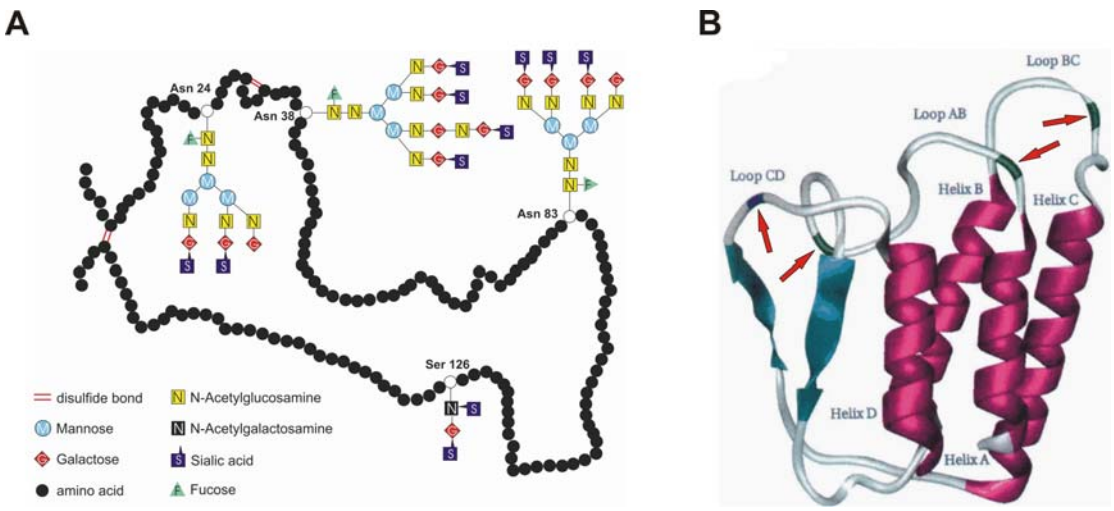


Figure 1. Structure of erythropoietin. (A) A scheme of human erythropoietin molecule with amino acid backbone, carbohydrate chains and disulfide bonds. From http://www.robertsaunders.org.uk/ (B) Ribbon diagram of the predicted Epo tertiary structure. The four α-helices are labeled *A-D (magenta);* Loops between helices are named for the helices they interconnect. Two regions of extended structure which could form hydrogen bonds between *Loop AB* and *Loop CD* are also presented *(cyan)*. The arrows indicate glycosylation sites. Modified from (Boissel *et al.*, 1993).

4.1.2 Expression of erythropoietin

Erythropoietin production is under the control of hypoxia-inducible factor-1 (HIF-1) which is recognized as the major regulator of oxygen homeostasis in the body (Wang *et al.*, 1993; Fandrey, 2004). The stability of HIF-1 is regulated by prolyl hydroxylases (PHDs), whose activity depends on oxygen availability, thus serving as oxygen sensors (Semenza, 2001; Fandrey, 2004). In normoxia HIF-1α subunit is hydroxylated by the PHDs (Fig. 2), leading to von Hippel–Lindau protein binding and recruitment of the ubiquitin E3 ligase complex. As a result, HIF-1α is ubiquitinated and degraded in the proteosomes (Semenza, 2001; Fandrey, 2004). The transcriptional activity of HIF-1 is further suppressed by oxygen-dependent hydroxylation of asparagine residues by HIF-α-specific asparaginyl hydroxylase (factor-inhibiting HIF-1; FIH-1). The later prevents the binding of the transcriptional co-activator CBP/p300 to HIF-1α (Fig. 2) decreasing HIF-1-induced gene

transcription (Fandrey, 2004). In contrast, under hypoxic conditions the activity of PHDs and FIH-1 is suppressed, HIF-1α is not degraded and its cellular levels increase rapidly. Further, HIF-1α is translocated to the nucleus, where it dimerizes with HIF-1β subunit and HIF-1 heterodimer binds to hypoxia responsive elements (HRE; Fig. 2) residing in the 3'-flanking region of the Epo gene (Semenza, 2001; Fisher, 2003; Fandrey, 2004). Finally, the recruitment of the transcriptional co-activator CBP/p300 to the HIF-1-DNA complex, results in increased transcription initiation complex (TIC) formation and mRNA synthesis (Fig. 2) (Wang *et al.*, 1993; Semenza, 2001; Fisher, 2003; Fandrey, 2004). HIF-1 translocation to the nucleus and transcriptional activity are further upregulated by MAPK- or PI3K/Akt-induced phosphorylation and nuclear factor κB (Nf-κB) activation (Fandrey, 2004; Maiese *et al.*, 2008).

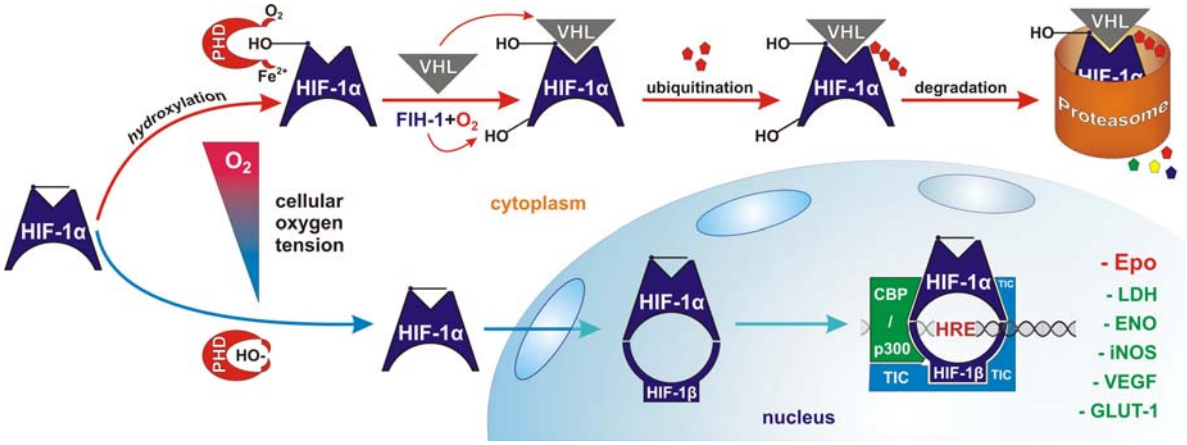


Figure 2. Regulation of HIF-1 activity and Epo expression by the cellular O_2 concentration. In the presence of oxygen HIF-1α is hydroxylated by the prolyl hydroxylases (PHDs) and factor-inhibiting HIF-1 (FIH-1). This leads to its binding to von Hippel–Lindau protein (VHL), ubiquitination and degradation in the proteasomes. During hypoxia, PhDs are inactivated, allowing HIF-1α to be translocated in to the nucleus. There, alpha and beta subunits of HIF-1 dimerize and bind to hypoxic responsive elements (HRE) sequences in the DNA. When transcriptional co-activator (CBP/p300) is recruited and transcription initiation complex formed, the expression of the HIF-1 responsive genes (including Epo) is triggered.

In addition to HIF-1, Epo expression is directly controlled by several other transcription factors including GATA 2, GATA 4 and Nf-κB. GATA-4 promotes Epo gene transcription while GATA-2 and Nf-κB suppress Epo production (Fandrey, 2004; Jelkmann, 2007; Noguchi *et al.*, 2008). The opposite role of Nf-κB in the

regulation of HIF-1 and Epo expression could represent a negative feedback mechanism controlling Epo levels. Interestingly, the inhibition of NO synthesis lowers Epo production by increasing GATA-2 DNA binding. GATA-2 and Nf-κB are activated also by the pro-inflammatory cytokines interleukin-1 (IL-1) and tumor necrosis factor α (TNF-α), and increased oxidative stress (Fandrey, 2004; Jelkmann, 2004).

Epo is mainly produced by hepatocytes during the fetal stage. After birth, almost all circulating cytokine originates from the peritubular, fibroblast-like interstitial cells located in the kidney cortex (Fisher, 2003; Fandrey, 2004; Noguchi *et al.*, 2008). Secondary sites of Epo production and secretion involve the central nervous system (CNS), female reproductive tract, placenta (Fandrey, 2004; Maiese *et al.*, 2008) and embryonic epicardium (Wu *et al.*, 1999; Stuckmann *et al.*, 2003). Epo mRNA expression was reported in several other organs and cell types including peripheral endothelial cells (ECs), insulin-producing cells, lungs, testis, myoblasts, and cardiac tissue (Fandrey, 2004; Maiese *et al.*, 2008). However not for all these locations Epo protein synthesis was unambiguously documented and the paracrine role of the cytokine is not well determined (Fandrey, 2004).

4.2 Erythropoietin receptor

4.2.1 Structure of erythropoietin receptor

Cloning of the Epo gene (Jacobs *et al.*, 1985; Lin *et al.*, 1985) and the synthesis of recombinant human Epo (rHuEpo), made possible the characterization of the erythropoietin receptor (Jelkmann *et al.*, 2008; Maiese *et al.*, 2008). Epo receptor (EpoR) gene is localized on chromosome 19 pter-q12 and is composed of 8 exons and 7 introns (Maouche *et al.*, 1991). The unprocessed human EpoR protein consists of 508 amino acids with a predicted molecular mass of approximately 56 kDa. Posttranslational processing includes cleavage of a 24 amino acid signal peptide, glycosylation and ubiquitination (Jelkmann *et al.*, 2008). The functional EpoR is a glycoprotein with molecular weight between 66 to 78 kDa (Fisher, 2003). The EpoR is a member of the type I, cytokine receptor superfamily. It has a single hydrophobic transmembrane spanning region, a cytoplasmic and an

extracellular region with an overall 15–35% homology to the other members of the cytokine receptor superfamily (Fisher, 2003; Jelkmann *et al.*, 2008).

The extracellular domain of the human EpoR contains 225 amino acids with 22 amino acids in the transmembrane domain and 236 amino acids in the cytoplasmic portion. The extracellular domain contains conserved cysteine residues and a “WSXWS” motif. This motif in the EpoR is critical for ligand binding, internalization and signal transduction. A high-affinity (kD ~ 1 nM) and a low-affinity (kD ~ 2 μM) binding sites for Epo has been demonstrated in the extracellular domain of the Epo receptor. Binding of Epo to the EpoR or to preformed EpoR dimers, results in conformational changes that trigger intracellular signaling (Lacombe *et al.*, 1999; Fisher, 2003; Jelkmann *et al.*, 2008).

Like all other receptors of the cytokine receptor superfamily EpoR has no kinase- or any other enzymatic domain in the cytoplasmic region. The membrane proximal region contains the Box 1 and Box 2 motifs with limited homology within the superfamily (Jelkmann *et al.*, 2008). The Box 1 motif of the EpoR and its flanking regions are necessary for the binding of the Janus family tyrosine protein kinase 2 (Jak2) to the receptor and for Jak2 activation (Lacombe *et al.*, 1999). Additionally, the EpoR cytoplasmic domains contain eight tyrosine residues which participate in the receptor-mediated signal transduction in the cytosol (Fisher, 2003).

4.2.2 Epo receptor expression

EpoR gene activation is under the control of several transcription factors, including the ubiquitous CACC-binding protein Sp-1 and erythroid-specific GATA-1 (Youssoufian *et al.*, 1993; Noguchi *et al.*, 2008). The onset of expression of EpoR by hematopoietic cells represents one of the earliest events after the commitment of the precursors to the erythroid lineage. The main physiological function of Epo/EpoR system is to regulate and potentiate bone marrow erythroid cell proliferation, differentiation, and survival (Youssoufian *et al.*, 1993; Fisher, 2003). However, EpoR signaling has been detected in a variety of non-hemopoietic tissues triggering extensive research to elucidate the putative non-hematopoietic effects of Epo (Noguchi *et al.*, 2008). EpoR mRNA, Epo binding sites and EpoR signaling have been found in the heart, blood vessels, kidneys, liver, gastrointestinal tissues, pancreatic islands, testis, female reproductive tract,

placenta and the brain (Sasaki *et al.*, 2000; Fisher, 2003; Sasaki, 2003; Li *et al.*, 2004; Depping *et al.*, 2005; Jelkmann *et al.*, 2008; Noguchi *et al.*, 2008).

4.2.3 Erythropoietin-dependent signal transduction

The Epo binding triggers dimerization and conformational changes of the EpoR (Lacombe *et al.*, 1999; Fisher, 2003; Jelkmann *et al.*, 2008; Maiese *et al.*, 2008). The intracellular Jak2 molecules, associated with the receptor pair come in to close proximity, transphosphorylate and activate each other (Fig. 3; 1-2). As the tyrosine residues in the cytoplasmic domain of the EpoR are phosphorylated, they serve as docking sites for intracellular proteins containing Src homology 2 (SH2) domains (Lacombe *et al.*, 1999; Fisher, 2003; Jelkmann *et al.*, 2008; Maiese *et al.*, 2008). After docking to the receptor complex, specific tyrosine residues of these proteins are then phosphorylated (Fig. 3; 3-5) by Jak2 (Lacombe *et al.*, 1999; Fisher, 2003; Jelkmann *et al.*, 2008; Maiese *et al.*, 2008).

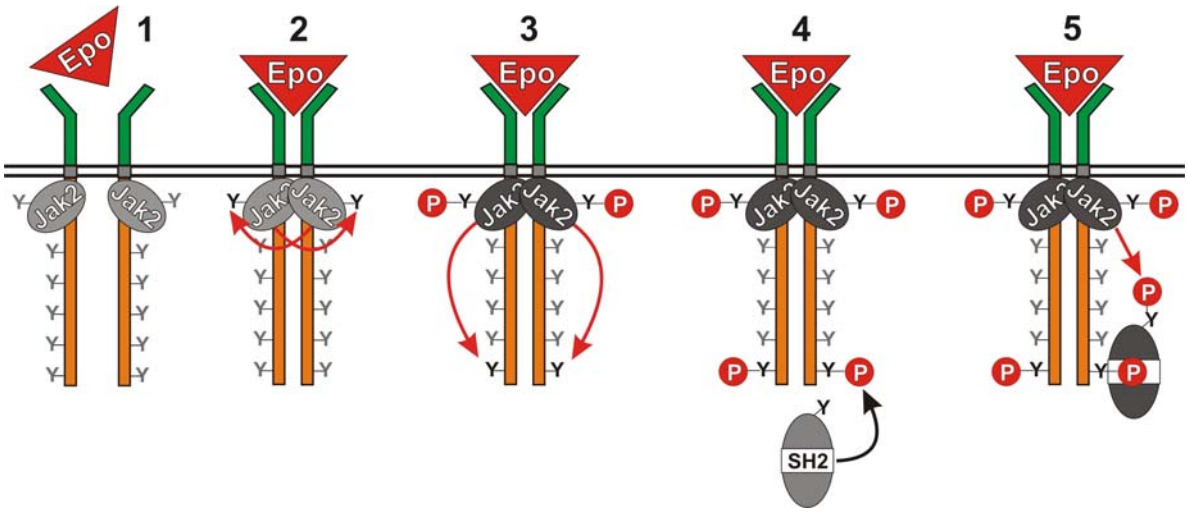


Figure 3. The first step in EPO activation of the receptor is dimerization (1); the pre-associated Jak2 kinases are in close contact and activated by transphosphorylation (2); the tyrosine residues of the EpoR are then phosphorylated (3, 4), providing docking sites for intracellular signaling proteins with SH2 domains (5).

EpoR homodimerization and activation upon Epo binding is crucial for hematopoietic function of the cytokine (Fisher, 2003). It was demonstrated that Epo or EpoR deficient mice, die around embryonic day E13.5 (Wu *et al.*, 1999; Noguchi *et al.*, 2008) suffering from severe anemia. Further examination revealed alterations in the development of the heart and brain prior to the onset of anemia,

including ventricular hypoplasia, increased apoptosis in the myocardium, endocardium and in the fetal brain, and a reduction in the number of cardiac myocytes and endothelial cells (Wu *et al.*, 1999; Noguchi *et al.*, 2008). These data suggest that Epo may act as an antiapoptotic and mitogenic factor and contribute to progenitor cell survival and proliferation during development.

In addition, increasing evidence suggests that the pleiotropic, tissue-protective effects of Epo in the brain and heart are dependent on the presence of cytokine receptor common β subunit (βcR) (Brines *et al.*, 2004; Jelkmann, 2007; Arcasoy, 2008). A physical association between the βcR and EpoR has been demonstrated in several experimental models and the requirement for βcR in the tissue-protective effect of Epo was shown using βcR knock-out ($\beta cR^{-/-}$) mice (Brines *et al.*, 2004; Arcasoy, 2008). In a spinal cord injury model, the recovery of motor function in response to Epo was absent in $\beta cR^{-/-}$ mice compared with strain-matched wild-type control animals (Brines *et al.*, 2004). Moreover, Epo failed to exert cardioprotection in primary cardiac myocytes isolated from $\beta cR^{-/-}$ mice subjected to staurosporine-induced apoptosis (Brines *et al.*, 2004). However the exact role of βcR in the hematopoietic and non-hematopoietic effects of Epo remains to be clarified.

EpoR activation leads to phosphorylation of a large number of proteins, among them several components of major signal transduction pathways as presented in Figure 4 (e.g. STAT-5, Ras/Raf/MAPK, PI3K/Akt and PLC-γ1/PKC signaling cascades) (Lacombe *et al.*, 1999; Fisher, 2003; Jelkmann, 2004; Foley, 2008; Jelkmann *et al.*, 2008; Maiese *et al.*, 2008). The signal is terminated when Jak2 is dephosphorylated by the hematopoietic cell phosphatase (HCP) which binds to the C-terminus of the intracellular domain of the EpoR (Klingmuller *et al.*, 1995).

STAT-5 signaling - The signal transducer and activator of transcription (STAT) proteins are direct substrates of Janus kinases and the STAT5 signaling pathway is involved in the Epo-induced effects on red blood cell differentiation and proliferation (Socolovsky *et al.*, 1999; Ratajczak *et al.*, 2001; Maiese *et al.*, 2008). The activation of Jak2 by Epo binding to its receptor, leads to tyrosine phosphorylation and dimerization of STATs (Fig. 4). The active STATs then translocate to the nucleus and bind to specific DNA sequences in the promoter

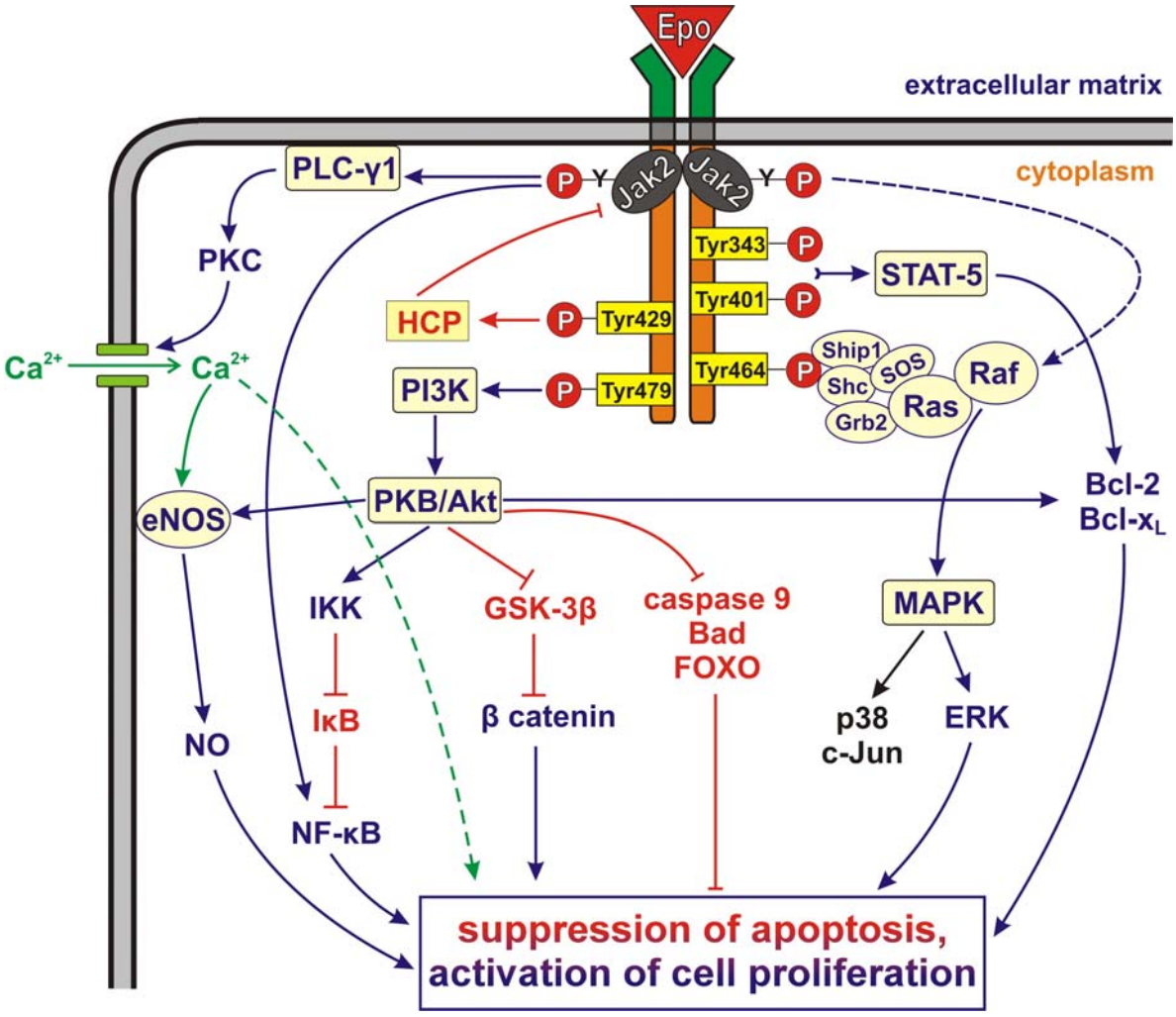


Figure 4. Major signaling pathways activated by the tyrosine phosphorylation of EpoR upon ligand stimulation.

regions of the responsive genes to lead to gene transcription, regulating many aspects of cell growth, survival and differentiation (Stephanou, 2004; Maiese *et al.*, 2008). The JAK2/STAT-5 pathway appears to be essential for the Epo-induced suppression of apoptosis of the erythroid precursors, since Epo induces Bcl-2 and Bcl-x_L anti-apoptotic factors through STAT-5 in erythroid progenitor cells (Fig. 4) (Wojchowski *et al.*, 1999; Jelkmann *et al.*, 2008). Induction of apoptosis can be observed in cells that lack STAT-5 (STAT-5$\alpha^{-/-}$/5$\beta^{-/-}$) function and STAT-5$\alpha^{-/-}$/5$\beta^{-/-}$ fetal liver erythroid progenitors, show higher levels of apoptosis and are less responsive to the presence of Epo (Socolovsky *et al.*, 1999). Recently it was reported that Epo-induced cardioprotection is accompanied by increased STAT-3 phosphorylation (Piuhola *et al.*, 2008). STAT-3 activation is involved in the ischemic preconditioning as it suppresses apoptosis and thereby is suggested to

reduce myocardial injury triggered by infarction or ischemia-reperfusion (Stephanou, 2004).

Ras-Raf-MAPK pathway - Activation of the EpoR leads also to tyrosine phosphorylation of Ship1 (SH2 inositol 5-phosphatase) resulting in recruitment of Shc (Src-homolgy and collagen) and Grb2 (growth factor receptor binding protein), members of the adaptor protein family (Mason *et al.*, 2000). The latter triggers binding to the receptor complex of SOS (son of sevenless) and GTPase Ras (Fig.4), and the sequential activation of the serine-kinase Raf (Jelkmann, 2004). Downstream targets of this signaling cascade are mitogen-activated protein kinases (MAPK), which include the extracellular signal-related kinases (ERKs), the c-Jun-amino terminal kinases, and p38 MAP kinase (Fig.4) (Mason *et al.*, 2000; Jelkmann, 2004). Additionally, it was shown that Jak2 can associate with Raf-1 and directly phosphorylate it (Chen *et al.*, 2004). Epo-induced activation of the Ras-Raf-MAPK pathway is involved in erythroid proliferation and differentiation, however the exact functional role of this signaling cascade in the hematopoietic and protective effects of Epo remain to be clarified (Sui *et al.*, 1998; Wojchowski *et al.*, 1999; Ratajczak *et al.*, 2001).

PI3K/Akt pathway - A third Epo-sensitive signaling pathway includes phosphatidylinositol-3 kinase (PI3K) and its downstream targets of which one is serine/threonine protein kinase B (Akt) (Fig. 4) (Wojchowski *et al.*, 1999; Fisher, 2003). In general, the activation of PI3K/Akt pathway prevents cellular apoptosis by regulation of transcription, stabilization of mitochondrial membrane potential ($\Delta\psi_m$), prevention of cytochrome c release, and blockade of caspase activity (Franke *et al.*, 2003). PI3K/Akt signaling plays a pivotal role in the cytoprotective action of Epo in both hematopoietic and non-hematopoietic tissues, since prevention of Akt phosphorylation abolishes the protective effects of the cytokine (Fliser *et al.*, 2006). When activated, Akt phosphorylates and inactivates BAD (a pro-apoptotic member of the Bcl-2 family), pro-apoptotic forkhead transcription factors (FOXO) and caspase 9 (Cross *et al.*, 1995; Cardone *et al.*, 1998). Furthermore, Akt phosphorylates and inactivates glycogen synthase kinase 3β (GSK-3β) (Cross *et al.*, 1995), allowing β-catenin to accumulate in the nucleus, thus promoting cellular survival and proliferation (Fig. 4). GSK-3β signaling was

recently reported to be essential for the cardioprotective effects of Epo, since the phosphorylation state of GSK-3β after *iv* administration of Epo or application of GSK-3β activity inhibitor, correlates with the reduction of infarct size (Nishihara *et al.*, 2006). Another downstream target of Akt is NF-kB (Fig. 4). Akt phosphorylates and inactivates the inhibitor of NF-kB (IkB) via activation of the I-kappa-B kinase (IKK). Thus, rescued from degradation, NF-kB translocates to the nucleus and activates the transcription of anti-apoptotic genes, including the inhibitors of apoptotic proteins (IAPs) and Bcl-x_L (Maiese *et al.*, 2008).

Ca^{2+} signaling – As shown on Figure 4, Epo binding to its receptor also induces tyrosine phosphorylation and activation of phospholipase C-γ1 (PLC-γ1) (Schaefer *et al.*, 1997). PLC-γ1 acts on $PI(4,5)P_2$ and generates two second messengers diacylglycerol (DAG) and $inositol(1,4,5)P_3$, that in turn activate PKC and regulate intracellular calcium levels (Schaefer *et al.*, 1997; Assandri *et al.*, 1999). Many data suggest the involvement of Ca^{2+} in erythroid growth and differentiation (for review see (Schaefer *et al.*, 1997; Cheung *et al.*, 2001)):

- Epo-induced murine erythroid colony growth is enhanced by the ionophore A23187 and inhibited by treatment with EGTA, a nonspecific chelator of Ca^{2+}.

- The increase in Ca^{2+} influx is an early and necessary step in the commitment to differentiation of murine erythroleukemia cells.

- Epo treatment of Friend virus-infected erythroid cells results in rapid changes in transmembrane Ca^{2+} flux.

- The significant rise in intracellular calcium stimulated by Epo is observed at later (day 10) but not earlier (day 7) stages of human BFU-E differentiation, when EpoR expression in the erythroid precursors is increased (see section 5.1.1).

The effect of Epo on cellular Ca^{2+} levels is mediated through a voltage-independent ion channel permeable to calcium and dependent on tyrosine phosphorylation, and the G-protein α-subunit Giα2 (Schaefer *et al.*, 1997; Cheung *et al.*, 2001). In addition, Epo was shown to affect Ca^{2+} homeostasis in neuronal cells expressing EpoR (Ghezzi *et al.*, 2004). However, the reported data are controversial and Epo was shown to cause both an increase and decrease of Ca^{2+} influx in the different cell types (Schaefer *et al.*, 1997; Assandri *et al.*, 1999; Ghezzi *et al.*, 2004). It is known that Ca^{2+}-dependent and phospholipid-dependent protein kinase C isoforms are involved in the regulation of neuronal (Simons, 1988) and

myocardial function (Bers, 2008), as well as in the control of apoptosis (Mattson *et al.*, 2003), but the role of the PLC-PKC-Ca^{2+} pathway in the tissue-protective effects of erythropoietin is not clear (Schaefer *et al.*, 1997; Ghezzi *et al.*, 2004; Fliser *et al.*, 2006).

4.2.4 Nitric oxide and erythropoietin

During the last years endothelial type nitric oxide synthase (eNOS) emerged as an important downstream target of Epo, regulated by at least two Epo-sensitive signaling cascades: PI3K/Akt and PLC/PKC/Ca^{2+} pathways (Fig. 4). Epo treatment results in elevated eNOS expression and activity in endothelial cells and cardiomyocytes via PI3K/Akt-mediated phosphorylation of eNOS at serine-1177 residue (Ser-1177) (Kanagy *et al.*, 2003; Beleslin-Cokic *et al.*, 2004; Burger *et al.*, 2006; Santhanam *et al.*, 2006). In addition, Epo may directly activate Ca^{2+}-sensitive NOSes (both eNOS and nNOS) in the rat hippocampus by increasing the opening probability of voltage-gated Ca^{2+} channels, thus triggering Ca^{2+} uptake (Yamamoto *et al.*, 2004). However, the initial reports of the potential effect of Epo on NO production were controversial. Some authors described an increased NO synthesis and enhanced expression of endothelial type NO synthase (eNOS) in venous and arterial endothelial cells after prolonged administration of Epo (Wu *et al.*, 1999; Banerjee *et al.*, 2000). Others found no change (Lopez Ongil *et al.*, 1996) or a decline in NO production and eNOS protein levels (Wang *et al.*, 1999). The diverging effects of Epo on the vasculature are most probably due to the Epo-induced activation of cyclooxygenase and production of endotheline-1 (ET-1) (Wada *et al.*, 1999). Cyclooxygenase synthesizes not only vasoconstrictor prostanoids but also superoxide anion (Katusic *et al.*, 1989), which deactivates endothelium-derived NO (Pacher *et al.*, 2007). In addition ET-1 is capable to reduce eNOS protein expression via the PKC-dependent pathway (Ramzy *et al.*, 2006). The balance between the two opposite processes, NO production and cyclooxygenase activation, depends strongly on the experimental conditions and Epo dosage, and defines the outcome of Epo application (Jie *et al.*, 2006).

NO-mediated effects of Epo on the vasculature include improvement of the endothelium-dependent modulation of the vascular tone, reendothelialization and neovascularization (Heeschen *et al.*, 2003; Urao *et al.*, 2006; d'Uscio *et al.*, 2007; Noguchi *et al.*, 2008; Santhanam *et al.*, 2008). In cardiomyocytes NO act as an

anti-apoptotic factor through a cGMP-dependent increase of Bcl-2 expression and an inhibition of L-type Ca^{2+} channels, and S-nitrosylation followed by inhibition of caspases, transcription activator protein 1 (AP-1) and tissue transglutaminase (for review see (Razavi *et al.*, 2005)). NO-induced neuroprotection is also mediated by soluble guanylyl cyclase upregulation (activation of Akt-pathway and cyclic-AMP-responsive-element-binding protein (CREB)) and S-nitrosylation (inhibition of caspase activity and Ca^{2+} influx trough NMDA receptors) (for review see (Calabrese *et al.*, 2007)).

Along with the effects described above, NO prevents H_2O_2 generation, serving as a scavenger of $*O_2^-$ as well as of *OH (Moncada *et al.*, 1991; Pacher *et al.*, 2007). Excessive oxidative stress and Ca^{2+} overload are one of the main causes of necrotic cell death (Zong *et al.*, 2006). The antioxidant properties of NO and its involvement in intracellular Ca^{2+} regulation are features making nitric oxide a potent suppressor of necrosis.

5. CHARACTERIZATION OF ERYTHROPOIETIN INTERACTION WITH MOUSE ERYTHROCYTES

5.1 Erythrocytes and erythropoietin

Erythrocytes are highly specialized cells that carry oxygen from the lungs to the peripheral tissues and allow a counterflow of carbon dioxide from the periphery to the lungs. The mature mammalian RBCs are flexible biconcave disks lacking cell nucleus, subcellular organelles and thus the ability to synthesize proteins. The size of erythrocytes and their life span vary between species, being 120 days in humans and 40 days in mice.

5.1.1 Erythroid development, Epo and EpoR

The expression of EpoR during the erythroid development, schematically presented on Figure 5 is transient (Wickrema *et al.*, 1992; Youssoufian *et al.*, 1993; Fisher, 2003).

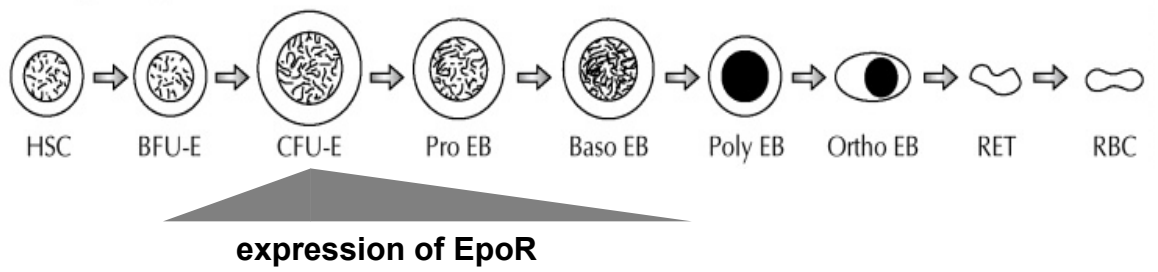


Figure 5. Expression of erythropoietin receptor during erythroid development; HSC – hematopoietic stem cell, BFU-E – burst-forming units-erythroid, CFU-E – colony-forming units-erythroid, Pro EB – proerythroblast, Baso EB – basophilic erythroblast, Poly EB - polychromatic erythroblast, RET – reticulocyte, RBC – mature red blood cell.

The earliest stage of erythroid progenitors expressing EpoR is BFU-E (burst-forming units-erythroid) (Broudy *et al.*, 1991; Wickrema *et al.*, 1992). EpoR mRNA and protein levels are low in BFU-E (between 100-200 receptors/cell), reaching maximum at CFU-E (colony-forming units-erythroid) stage, with about 1000 receptors/cell and gradually decreasing in the course of terminal differentiation (Fig. 5). The primary role of Epo in erythroid development is inhibition of apoptosis and enhancement of cellular proliferation, thus increasing

the numbers of hematopoietic progenitors in the bone marrow (Fisher, 2003; Jelkmann, 2007; Foley, 2008). Hematopoietic effects of Epo are mediated by several signaling pathways reviewed in chapter 4.2.3, namely STAT-5, PI3K/Akt, Ras/Raf/MAPK and Ca^{2+} signaling (Schaefer *et al.*, 1997; Jelkmann, 2007; Foley, 2008).

5.1.2 Evidence for Epo-binding to reticulocytes and mature erythrocytes

As shown in the previous chapter EpoR mRNA and protein levels decrease gradually with terminal erythroid development. Moreover, 24-48 h after their release into the circulation, reticulocytes undergo a process of vesicles/exosomes release, called shedding which convert them to mature erythrocytes (Snyder *et al.*, 1985; Johnstone *et al.*, 1987; Blanc *et al.*, 2005). During this transition the remaining organelles (mitochondria and ribosomes) are removed, along with many membrane bound proteins such as nucleoside-, glucose- and amino acid transporters, Na^+/K^+-ATPase and transferin receptors (Johnstone *et al.*, 1987; Blanc *et al.*, 2005). The initial studies made in the beginning of 90-s, using radiolabeled Epo as a marker for Epo binding, couldn't detect EpoR in reticulocytes and mature erythrocytes and these cells were therefore considered to be Epo-insensitive (Broudy *et al.*, 1991; Wickrema *et al.*, 1992). However, binding of Epo to erythrocyte membranes was described by Baciu and Ivanof (Baciu *et al.*, 1983). The authors reported that *in vitro* incubation of rat erythrocytes isolated from well-oxygenated blood with hypoxic serum containing high amounts of Epo resulted in reduction of the serum cytokine levels. In contrast, RBCs isolated from hypoxic blood had markedly decreased ability to bind Epo (Baciu *et al.*, 1983). Twenty years later Myssina and colleagues (Myssina *et al.*, 2003), using radiolabeled cytokine showed that human erythrocytes are able to specifically bind erythropoietin. The authors reported the presence of about 6 Epo binding sites/cell assuming equal number of binding sites per erythrocyte independent of its age. The affinity of the Epo binding sites was found to be in the range of that previously reported for the classical EpoR (Myssina *et al.*, 2003).

5.1.3 Epo-induced responses in reticulocytes and mature erythrocytes

Publications on the Epo-induced responses in mammalian red blood cells outnumber those on Epo binding to the erythrocytes. Epo treatment of rat

erythrocytes was shown to cause an increase in ribose (Baciu *et al.*, 1983) and glucose uptake (Ghosal *et al.*, 1987). Administration of rhEpo to healthy human subjects was reported to increase the intracellular 2,3-diphosphoglycerate (2,3-DPG) content of RBCs (Birgegard *et al.*, 2001). These changes of 2,3-DPG levels were not secondary to the increase in reticulocyte count but most likely reflected direct action of the cytokine on mature erythrocytes.

Starvation of rats caused reduction of endogenous Epo plasma levels and was accompanied by impaired Mg^{2+}-, Ca^{2+}- and Na^{+}/K^{+}-ATPase activity of erythrocytes (Chakraborty *et al.*, 1986). The normal function of the ATPases could be restored by Epo supplementation (Chakraborty *et al.*, 1986). In addition, it was shown that *in vitro* exposure of human RBCs to Epo resulted in a dose-dependent inhibition of Ca^{2+}-permeable cation channels and suppression of osmotic hemolysis (Myssina *et al.*, 2003).

Finally, Epo exerted antioxidant properties as it enhanced RBCs defense systems, thus preventing lipid oxidation and promoting erythrocyte membrane integrity (Chakraborty *et al.*, 1988; Chattopadhyay *et al.*, 2000). The impairment of the several key enzymes regulating redox state in RBCs (including superoxide dismutase, catalase, glutathione peroxidase and glutathione reductase) caused by animal starvation or oxidative stress, could be surmounted by *in vivo* and *in vitro* Epo administration (Chakraborty *et al.*, 1988; Chattopadhyay *et al.*, 2000).

Erythrocyte metabolism, ion balance and redox state are key factors in control of red cell rheology, oxygen caring function and life span (Baskurt *et al.*, 2003). Responses of the circulating erythrocytes to Epo may therefore represent an acute adaptive response allowing an improvement of tissue perfusion and oxygen delivery, before the upregulation of erythropoiesis occurs.

5.2 General objectives and experimental model validation

This study was designed to characterize in details the Epo binding to erythrocytes depending on their age. We aimed to identify the Epo-sensitive signaling pathways and downstream targets of Epo action in mammalian erythrocytes. For the reasons mentioned above we particularly focused on the effects of Epo on the function of eNOS in erythrocytes. We furthermore investigated the effects of Epo on the cell volume and ion transport across the red cell membrane. To solve the tasks we have chosen mouse erythrocytes exposed to either endogenous mouse or human recombinant Epo (rhEpo) *in vivo* and *in vitro* for the following reasons:

- The existence of various transgenic mouse strains, particularly tg6 mouse strain with systemic overexpression of erythropoietin and homozygous eNOS deficient animals ($eNOS^{-/-}$) could be used to reveal the possible effects of Epo on the circulating RBCs.

- The amino acid sequence of the human EpoR is 82% identical to that of the murine protein and mice were extensively used for characterization of EpoR signal transduction and hematopoietic properties of rhEpo (Youssoufian *et al.*, 1993; Spivak *et al.*, 1996). It is now clear, that rhEpo acts on mice in the same way as in humans by increasing hematopoiesis (Vogel *et al.*, 2003; Bogdanova *et al.*, 2007).

- endothelial type nitric oxide synthase as well as an array of ion transporters, including Na^+/K^+-ATPase, K^+-Cl^--cotransporter (KCC) and Na^+-K^+-Cl^--cotransporter (NKCC) are present in human and mouse red cells (Delpire *et al.*, 1994; Kleinbongard *et al.*, 2006; De Franceschi *et al.*, 2007; Lambert *et al.*, 2008).

- The time course and kinetics for plasma erythropoietin (endogenous) in men and mice subjected to hypoxia is similar (Abbrecht *et al.*, 1972).

All these features make mouse RBCs a suitable model for investigation of the putative mechanisms of Epo-induced effects in mature erythrocytes.

5.3 Own research (based on Papers 1 and 2)

5.3.1 Characterization of Epo binding to mouse erythrocytes

Radioactively labeled rhEpo (^{125}I-Epo) is widely used for characterization of Epo binding to the erythroid precursors in the bone marrow or hematopoietic cell lines and the experimental protocols and assay sensitivities are well established (Fraser *et al.*, 1988; Sawada *et al.*, 1990; Broudy *et al.*, 1991; Wickrema *et al.*, 1992; Spivak *et al.*, 1996). Incubation of mouse erythrocytes with different concentrations of ^{125}I-Epo revealed that the cytokine binds specifically to the red blood cells (Paper 1). Specificity of the binding was proven as excessive amounts of "cold" non-labeled Epo could successfully compete the ^{125}I-Epo from its binding sites. The remaining residual binding of the radiolabeled cytokine was considered as nonspecific and was subtracted from the total binding values. Analysis of the dose-dependence of specific ^{125}I-Epo binding, revealed a single class of binding sites with a dissociation constant (K_D) of 58.3 ± 11.1 pmol/l. The K_D values we have obtained were within the range of affinity of the classical EpoR to erythropoietin reported in the literature (30 - 330 pmol/l (see (Broudy *et al.*, 1991; Wickrema *et al.*, 1992; Spivak *et al.*, 1996; Myssina *et al.*, 2003)). The wide scatter of the K_D values reveals a broad tissue- and species-specific variation of the EpoR properties.

Similar technique used to track Epo binding to human erythrocytes revealed the presence of 5-6 Epo binding sites per cell, assuming that the number of specific Epo binding sites is equal for each erythrocyte (Myssina *et al.*, 2003). If the same assumption is applied to our data, similar numbers may be obtained (5-6 Epo binding sites per mouse erythrocyte). However it is known that many properties of the RBCs are altered during their maturation and aging (Johnstone *et al.*, 1987; Suzuki *et al.*, 1989; Lutz *et al.*, 1992; Waugh *et al.*, 1992). Therefore we characterized the age-dependence of the Epo-binding in mouse erythrocytes. We divided the isolated mouse erythrocytes into three fractions according to their age using two major markers: density (Lutz *et al.*, 1992) and the band 4.1a/band 4.1b protein ratio (Suzuki *et al.*, 1989). The obtained sub-populations were considered as reticulocytes + young erythrocytes (group 1), mature erythrocytes (group 2) and senescent erythrocytes (group 3). We found that reticulocytes and young erythrocytes, representing about 2% of the RBC population have markedly higher

numbers of Epo-binding sites in comparison to adult and old RBCs (~85% and ~17% of the total RBC population respectively), namely 105 ± 8 binding sites per cell for group 1, 4 ± 1 for group 2 and 2 ± 1 for group 3.

5.3.2 Downstream targets of Epo in mouse erythrocytes

Erythrocytes retain a diversity of signaling components present in erythroid precursors and nonerythroid cells (Cohen *et al.*, 1992; Minetti *et al.*, 1997). Some of the elements of signaling pathways may be vestiges of cascades critical to the erythroid development but no longer needed in the mature RBCs (STATs, MAPK). Other members of signaling cascades like PKA, PI3K, Akt, PKC, sGC, Ca^{2+}/calmodullin dependent kinase, *src* kinases and PLC, are likely involved in sensing of the alterations in the cellular environment and the corresponding adjustment of the oxygen delivery and homeostasis (Minetti *et al.*, 1997; Barvitenko *et al.*, 2005). Some of these pathways, e.g. PI3K/Akt cascade are known to be Epo-sensitive in the erythroid precursor cells. We thus monitored if recombinant human Epo treatment of mouse erythrocytes affects the activity of PI3K/Akt cascade (Paper 1).

Epo administration caused a significant increase in Akt phosphorylation, indicating that Epo binding to erythrocytes triggers activation of the “classical” Epo-sensitive signal-transduction cascade. Akt has a variety of downstream targets in erythroid precursors and non-erythroid cells including endothelial type nitric oxide synthase. Presence of eNOS in human and mouse erythrocytes (RBC-eNOS) was reported recently (Kleinbongard *et al.*, 2006). The activity of the RBC-eNOS is comparable to that observed in conventional endothelium-derived eNOS (Kleinbongard *et al.*, 2006). It was shown that insulin activates RBC-eNOS by phosphorylation of the enzyme at Ser-1177 via the PI3K/Akt pathway (Kleinbongard *et al.*, 2006). Therefore, we considered RBC-eNOS as a putative downstream target for Epo-induced PI3K/Akt activation and monitored the phosphorylation of RBC-eNOS at Ser-1177 in the mouse RBCs treated with rhEpo. In addition we estimated the effect of Epo on RBC-eNOS activity in the presence or absence of PI3K and Akt blockers (see chapter 5.3.3). The obtained results confirmed that Epo along with insulin may cause an increase in phosphorylation of the RBC-eNOS acting via PI3K/Akt signaling cascade (Fig 6, Paper 1).

5.3.3 Characterization of Epo effect on erythrocyte NO production

The following studies focused on the Epo-induced regulation of NO production by using NO_2^-/NO_3^- as markers of RBC-eNOS function. These anions are stable products of NO oxidation (Fig. 6) and are therefore considered as reliable indicators of NO generation in biological systems (Kleinbongard *et al.*, 2003). We measured NO_2^-/NO_3^- levels in the cells (Dejam *et al.*, 2005) and incubation medium (Kleinbongard *et al.*, 2003) to estimate RBS-eNOS activity in erythrocytes, treated with various doses of rhEpo for various time periods (Paper 1). Epo treatment resulted in a time- and dose dependent increase in erythrocyte NO production in comparison to control (non-treated) cells (Fig. 6). Differences in NO_2^-/NO_3^- levels between the Epo-treated and control erythrocytes were detected already 30 min after the cytokine application, reaching steady state after 1 h of treatment. When Epo was applied in physiological (1-10 U/ml) and therapeutic (10-100 U/ml) doses, a bell-shaped dose dependence of NO production was observed with a peak at 10 U/ml rhEpo. Of note, similar bi-phasic dose-dependent induction of NO production by Epo was reported in endothelial cell lines (Beleslin-Cokic *et al.*, 2004).

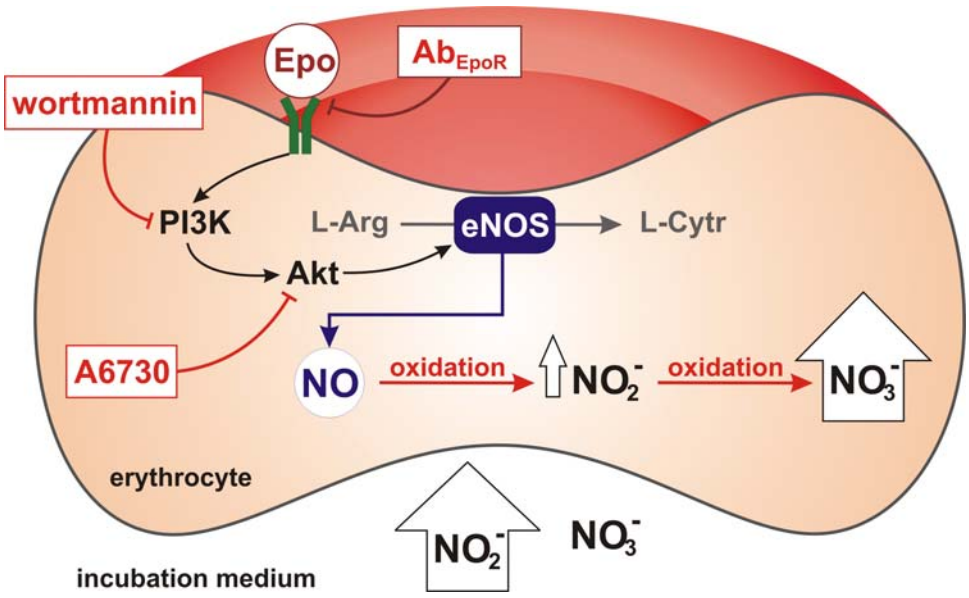


Figure 6. Mechanism of Epo-induced regulation of RBC-eNOS. Our data revealed the presence of a single class of Epo binding sites in mouse erythrocyte membrane, the number of which depends on the cell age. Epo binding triggers activation of PI3K/Akt pathway, phosphorylation of RBC-eNOS and up-regulation of NO production. The activity of RBC-eNOS and NO production rate were evaluated by monitoring nitrite (NO_2^-) and nitrate (NO_3^-) levels in the cells and the incubation

medium. rhEpo treatment resulted in increase in NO_2^-/NO_3^- cellular content and elevation of NO_2^- levels in the incubation medium.

The observed effects of Epo on NO production were erythrocyte-specific and independent of the presence of trace amounts of leukocytes in the red blood cell suspension. Further experiments revealed that rhEpo treatment of RBCs of eNOS knockout animals does not alter NO_2^-/NO_3^- levels in the cells or incubation medium. The obtained results confirm the specific, direct and acute modulatory effect of rhEpo on the NO production (Paper 1).

The Epo-induced increase of NO production was a direct consequence of the activation of the PI3K/Akt pathway because it could be completely abolished by pre-treatment of the RBCs with PI3K inhibitor wortmannin, Akt blocker A6730 or EpoR antibody (Fig. 6). No changes in NO_2^-/NO_3^- levels were observed in Epo-treated erythrocytes of the $eNOS^{-/-}$ mice (Paper 1).

5.3.4 Evidence for Epo-induced regulation of NO_2^- transport across erythrocyte plasma membrane

In erythrocytes pretreated with N^G-mono-methyl-L-arginine (L-NMA) to block RBC-eNOS, the Epo-induced increase in cellular NO_3^- content was completely blocked (Fig. 7).

Figure 7. Erythropoietin affects NO_2^- distribution between the cells and medium. When de novo NO production was blocked, rhEpo treatment resulted in a small, but statistically significant increase in NO_2^- levels in the incubation medium with a simultaneous depletion of RBC NO_2^- content.

NO_2^- accumulation in the medium triggered by Epo was only partially suppressed and was significantly exceeding that in the untreated RBCs (compare Figures 6 and 7). Furthermore, in the presence of L-NMA Epo treatment resulted in NO_2^- depletion of the erythrocytes in comparison to non-treated cells (Fig. 7). Our data imply that Epo facilitates NO_2^- release into the incubation medium independently from its effect on *de novo* NO synthesis.

5.3.5 Epo treatment modulates the redox state of mouse erythrocytes

Along with other antioxidative enzymes such as SOD or catalase, eNOS is in control of the redox state in the cells. In the presence of saturating amounts of the substrate (L-arginine, L-Arg) and obligatory co-factors (tetrahydrobiopterin, flavin mononucleotide**,** flavin adenine dinucleotide, Ca^{2+}, calmodulin), eNOS generates predominantly NO (Fleming *et al.*, 2003; Mount *et al.*, 2006). Nitric oxide, when present in the physiological concentration range (nM-μM), serves as an important scavenger of superoxide anions ($*O_2^-$) and hydroxyl radicals (*OH), whereby contributes to the cellular redox balance (Moncada *et al.*, 1991; Pacher *et al.*, 2007). On the other hand, under conditions of substrate or cofactor deficiency or in response to atherogenic stimuli (e.g. hyperglycemia and high LDL plasma levels), eNOS generates $*O_2^-$ rather than NO (Channon *et al.*, 2002; Munzel *et al.*, 2005). Mouse erythrocytes were not an exception to the rule and omission of L-Arg in the incubation medium resulted in an increased $*O_2^-$ production, causing oxidative stress (Paper 1).

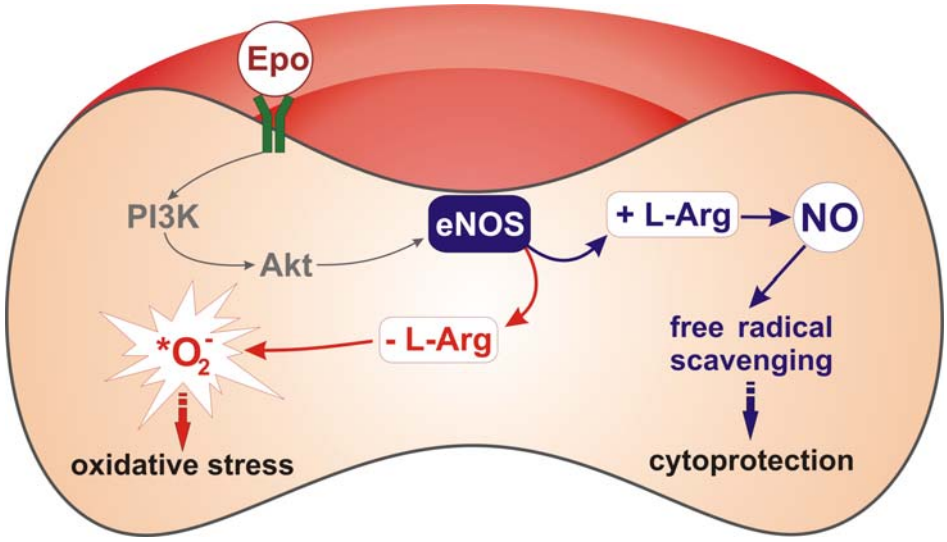


Figure 8. Putative effect of Epo-induced up-regulation of RBC-eNOS activity on the cellular redox state. In the absence of the enzyme substrate (red arrows), Epo treatment resulted in enhanced

$*O_2^-$ and increased oxidative stress. When Epo was applied in therapeutic doses and in the presence of L-Arg (blue arrows), the cellular redox state was shifted to more reduced thus protecting the cells from oxidation.

Epo-induced activation of RBC-eNOS in the absence of extracellular L-Arg further facilitated $*O_2^-$ generation and caused intracellular glutathione depletion (Fig. 8). In contrast, when the incubation medium was supplemented with L-Arg, the cellular redox state was maintained during the administration of physiological doses of Epo (1-10 U/ml), whereas the application of 100 U/ml rhEpo (therapeutic dose) shifted cellular redox potential to more reduced, thus making the cells more tolerant to oxidative stress (Fig. 8).

5.3.6 Chronic and acute effects of Epo on ion transport in mouse erythrocytes *in vivo* and *in vitro*

We used three different experimental models to characterize the effects of Epo on ion transport and volume regulation in mouse erythrocytes: chronic *in vivo* model, using a transgenic mouse strain with systemic overexpression of rhEpo (tg6 mice) (Paper 2); acute *in vivo* model, using wt mice exposed to hypoxia (8% O_2) for 4 h to induce endogenous Epo expression (unpublished data); and acute *in vitro* model, using erythrocytes isolated from wt animals, incubated with 1 U/ml rhEpo at room temperature (unpublished data).

I. Chronic effect of Epo on RBC ion transport

Overexpression of rhEpo in the transgenic mouse strain tg6 leads to an increase in Epo plasma levels up to 12-fold over the wt siblings (Vogel *et al.*, 2003). Permanently high plasma Epo concentrations result in pathologically up-regulated erythropoiesis and a doubling of the hematocrit (87.8 ± 2 % in tg6 vs. 43.1 ± 3 % in wt), the hemoglobin concentration (26 ± 1 g/dL in tg6 vs. 14 ± 1 g/dL in wt) and the reticulocyte counts (3.4 ± 1.6 % in tg6 vs. 1.7 ± 0.6 % in wt) (Vogel *et al.*, 2003). It was found that the animals adapt to the excessive erythrocytosis by two major mechanisms (Vogel *et al.*, 2003): 1) enhanced NO production in the vascular endothelium; 2) reduction in the blood viscosity. The latter is mostly due to the increased mean corpuscular volume and enhanced flexibility of tg6 erythrocytes (Vogel *et al.*, 2003).

A further characterization of tg6 erythrocyte properties revealed that the cellular K^+ and water content is higher in comparison to RBC from wt animals (Paper 2). In accordance to this, the unidirectional K^+ fluxes across the plasma membrane showed an enhancement of the active (ouabain-sensitive, Na^+/K^+-ATPase-mediated) K^+ influx in transgenic erythrocytes, coupled with a partial suppression of the passive (bumetanide-sensitive, NKCC-mediated) K^+ efflux. The accumulation of potassium and water in tg6 erythrocytes resulted in a decrease in the mean corpuscular hemoglobin concentration, lower internal viscosity and together with the higher surface-to-volume ratio, to an increased red cell flexibility as observed by osmotic gradient ektacytometry (Paper 2).

However, the direct effect of Epo on ion transporters in mature erythrocyte is not the only explanation for the observed alterations of tg6 RBC rheological properties. As mentioned above, the number of reticulocytes in tg6 mice is doubled (Vogel *et al.*, 2003) and the increased amount of young and more deformable cells will conduce to the observed changes in RBC rheology. The high NO levels in the circulating blood of tg6 animals would additionally contribute to the regulation of the red cell deformability and help the animal to cope with the excessive erythrocytosis (Vogel *et al.*, 2003).

II. Acute effect of hypoxia on RBC ion transport

The availability of a transgenic mouse model of chronic Epo overexpression provides us with valuable information on the possible adaptive mechanisms of the organisms coping with the excessive erythrocytosis. Unfortunately, the evidence for a direct, Epo-induced regulation of the ion transport across the erythrocyte membrane is vague. Therefore, we used a physiologically more relevant model to characterize the acute effects of endogenous Epo on Na^+/K^+-ATPase and NKCC activity in wt mice (C57Bl/6 mouse strain).

As mentioned in the introduction, Epo expression is enhanced under low oxygen conditions. We induced endogenous Epo production by subjecting the animals to normobaric hypoxia. The exposure of wt mice to 8% O_2 for 4 h resulted in an increase in plasma Epo levels up to those observed in the transgenic animals (219 ± 14 mU/ml for wt mice after hypoxia vs. 259 ± 79 mU/ml for tg6 animals). 24 h after hypoxic stimulus plasma Epo was still significantly elevated in comparison to normoxic controls (Fig. 9A). A trend for increased hematocrit

observed immediately after the hypoxic exposure, represents diuresis-induced reduction in plasma volume and/or release of red cells from the spleen rather than *de novo* erythrocyte production. However 24 hours after returning the animals back to normoxia, a significant rise of hematocrit levels was observed (Fig. 9B). The changes in hematocrit could not be explained solely by Epo-induced elevation of reticulocyte and mature RBC numbers, since a lag period of about 1.7 days is needed for detectable stimulation of erythropoiesis (Krzyzanski *et al.*, 2007).

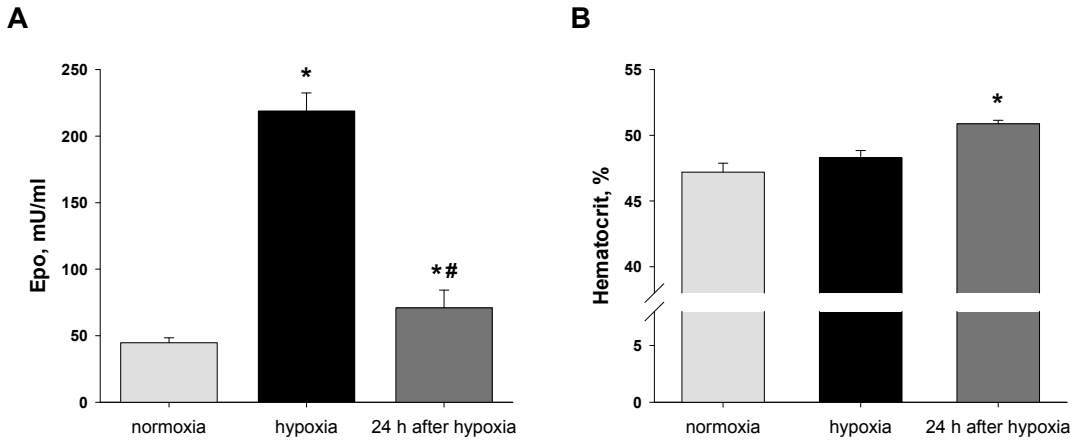


Figure 9. Plasma Epo levels (left graph, A) and hematocrit (right graph, B) in wt mice. The animals were exposed to 8 % O_2 for 4 h and than returned to normoxia. Plasma Epo and hematocrit were measured before (light-grey bars), immediately after (black bars) or 24 h after the hypoxic exposure (dark-grey bars). **P* ≤ 0.05 vs. normoxia; #*P* ≤ 0.05 vs. hypoxia, n=6.

We isolated erythrocytes from the wt animals exposed to hypoxia and measured the unidirectional K^+ influx across the plasma membranes by ^{86}Rb as a radioactive tracer for potassium. The activity of the Na^+/K^+-ATPase, Na^+-K^+-Cl^--cotransporter (NKCC), and ouabain-bumetanide-resistant K^+ influx was estimated by application of specific inhibitors as described in Paper 2.

A trend for an increase in total potassium influx was observed after hypoxic exposure (Fig. 10). However, the differences found were not statistically significant. Hypoxic responses of the Na^+/K^+-ATPase and NKCC resembled those observed in tg6 mice. The active K^+ influx was increased after the exposure of wt mice to low oxygen (Fig. 10). Concomitantly, the passive potassium flux through NKCC was inhibited by hypoxia. Hypoxia-induced changes in the activity of the ion

transporters were maintained over 24 hours after the animals were returned to normoxia (Fig. 10).

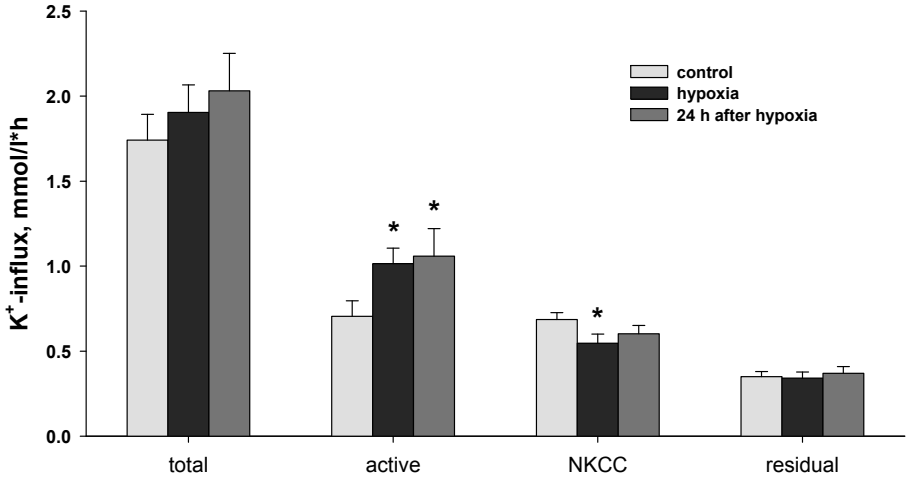


Figure 10. Hypoxia induced alteration of ion transport in wt erythrocytes. Exposure of wt mice to 8% O_2 for 4 h resulted in up-regulation of the active (ouabain-sensitive, Na^+/K^+-ATPase) trans-membrane K^+ flux coupled with a suppression of the passive (bumetanide-sensitive, NKCC) flux. **P* ± 0.05 vs. normoxic controls (light-grey bars), n = 6.

The observed activation of potassium uptake through Na^+/K^+-pump and the decrease of its leakage through NKCC should lead to an elevation of the cation and water content in the erythrocytes as in the cells of tg6 mice. The latter was not assessed due to the technical limitations (relative small amount of blood in mice, which does not allow performing of a large number of assays simultaneously). However, when occurring, hypoxia-induced erythrocyte swelling would contribute to the observed acute increase in hematocrit after the hypoxic treatment.

III. *In vitro* effect of rhEpo on RBC ion transport

To investigate whether the changes in RBC ion transport observed in Epo-overexpressing animals and wt mice exposed to hypoxia are a direct effect of Epo on erythrocytes, we used an *in vitro* experimental approach. RBCs isolated from wt animal were incubated for 30 min in the presence or absence of 1 U/ml rhEpo and potassium fluxes were measured. Similar to that in tg6 mouse erythrocytes and in red cells of hypoxia-treated animals, *in vitro* exposure of wt RBCs to rhEpo led to alterations in ion transporters activity. rhEpo caused a partial suppression of

the NKCC (Fig. 11). A trend for the activation of the Na^+/K^+-ATPase was observed, however the changes were not statistically significant (Fig. 11).

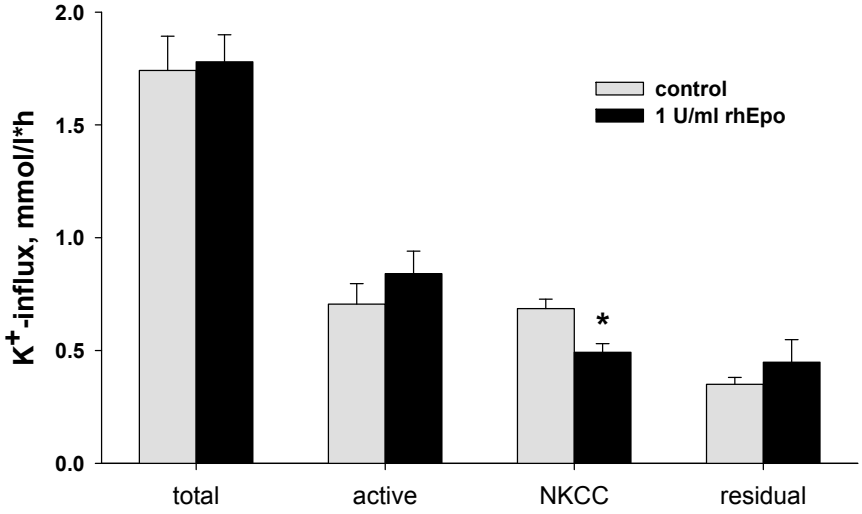


Figure 11. Effect of *in vitro* incubation of wt RBCs with rhEpo. Erythrocyte suspensions isolated from wt mice were pretreated with rhEpo (1 U/ml) for 30 min before the measurement of the K^+-influxes. Epo administration resulted in a pronounced inhibition of the passive potassium flux through NKCC. **P* ± 0.05 vs. corresponding control (grey bar), n = 6.

Further work is needed to characterize the dose dependence and kinetics of the Epo-induced regulation of ion transporters as well as the possible links between these effects and cytokine-triggered increase in NO production. On the one hand, ion transporters could be direct downstream targets of the Epo sensitive signaling cascades in mouse erythrocytes. On the other, it is known that NO and redox state are involved in the regulation of cellular ion homeostasis (see chapter 5.4). Interestingly, our preliminary experiments revealed that Epo does not affect ion transport in erythrocytes from $eNOS^{-/-}$ deficient mice. This suggests that ion transport regulation could be secondary to Epo-induced effects on RBC-eNOS activity or redox state (data not shown).

Overall, our results suggest that Epo is able to regulate ion transports through the erythrocyte membrane which affect the ion/water balance and cell volume. A concomitant up-regulation of the K^+ uptake through Na^+/K^+-ATPase and suppression of potassium leakage through NKCC would result in cation

accumulation in the cells with a corresponding increase in the water content and erythrocyte swelling (Fig. 12).

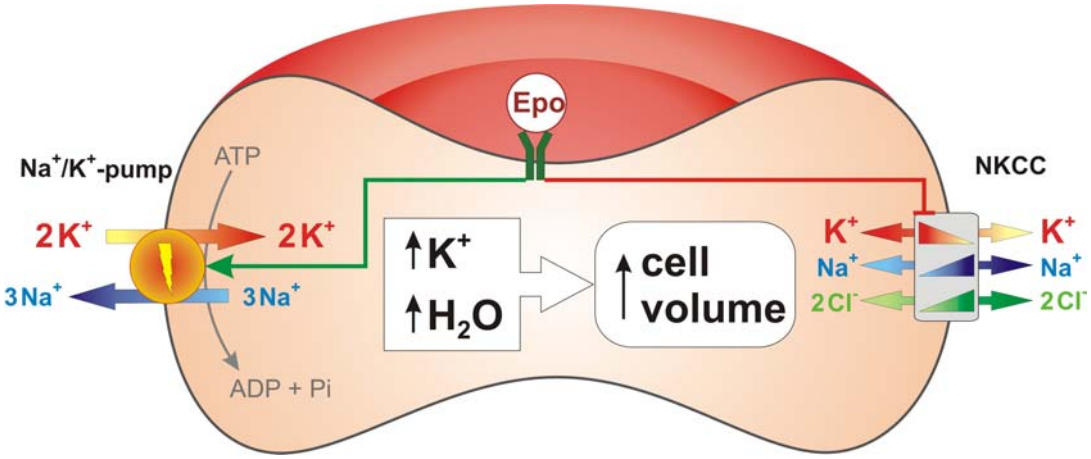


Figure 12. Schematic representation of the effects of Epo on erythrocyte ion transporters. The systemic overexpression of Epo (tg6 mice), hypoxia exposure (wt mice) and *in vitro* incubation of erythrocytes (wt mice) with rhEpo resulted in up-regulation of active K^+ uptake through the Na^+/K^+-pump and inhibition of cation leakage through NKCC. This in turn, leads to K^+ and water accumulation in the RBCs and increased cell volume.

5.3.7 Effect of Epo on glucose uptake in mouse erythrocytes

As mentioned in section 5.1.3, Epo was reported to regulate the uptake of monosaccharides by the erythrocytes (Baciu *et al.*, 1985; Ghosal *et al.*, 1987), thus potentially affecting the rate of glycolysis and ATP production, which in turn can modulate the function of the ion transporters. We have measured the uptake of radiolabeled D-glucose and ATP levels in erythrocytes from wt mice incubated for 30 min with 1 U/ml rhEpo (unpublished data). In accordance with the data reported for rat RBCs (Ghosal *et al.*, 1987), glucose uptake was increased in mouse red cells in response to rhEpo treatment (Fig. 13). Erythrocytes from tg6 animals, permanently exposed to a 12-fold higher plasma Epo concentration as compared to the wt animals, showed an elevated basal glucose uptake compared to wt controls. Consequently, glucose uptake could not be further activated by treatment of RBCs from tg6 mice with 1 U/ml rhEpo *ex vivo* (Fig. 13).

High glucose uptake by tg6 erythrocytes resulted in elevated intracellular ATP levels compared to those in wt RBCs (15.9 ± 1.145 nmol/ml tg6 RBCs vs. 13.3 ± 0.45 nmol/ml wt RBCs, $P \leq 0.05$, n = 16). Interestingly, *in vitro* incubation of

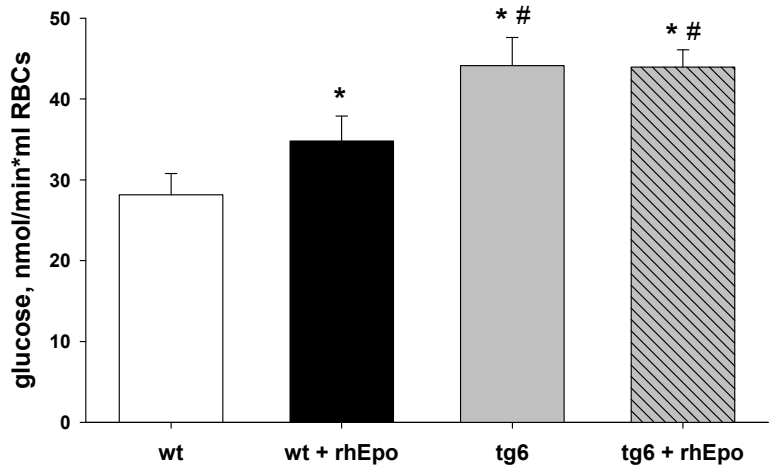


Figure 13. Glucose uptake in erythrocytes from wt and tg6 mice. Incubation of wt RBCs with 1 U/ml rhEpo for 30 min resulted in increase in glucose uptake (black bar) in comparison to non-treated control (white bar). The basal glucose influx (gray bar) was higher in tg6 erythrocytes comparing to wt control and the *in vitro* incubation with rhEpo (gray-dashed bar) had no further effect on glycolytic substrate uptake. **P* ≤ 0.05 vs. wt control (white bar), #*P* ≤ 0.05 vs. wt+rhEpo (black bar), n = 6.

wt erythrocytes with 1 U/ml rhEpo for 30 min had no effect on cellular ATP (data not shown). The inability of Epo to up-regulate the intracellular ATP levels may be due to the simultaneous activation of ATP-consuming processes (e.g. Na^+/K^+-ATPase activation) and insufficient duration of rhEpo exposure (30 min in wt animals vs. life-long one in the transgenic animals). Further experiments are required for a complete characterization of the kinetics, dose dependence and the mechanisms of Epo–induced up-regulation of glucose uptake and RBC metabolism.

5.4 Discussion I

5.4.1 Major findings

The primary physiological function of erythropoietin is to regulate RBC number for adequate oxygen supply to the tissues (Fisher, 2003). Expression of EpoR and Epo-induced responses in the erythroid progenitor cells are broadly studied and well characterized (Broudy *et al.*, 1991; Wickrema *et al.*, 1992; Tilbrook *et al.*, 1999). However, the presence of Epo binding sites on reticulocytes and mature erythrocytes was disputable. Our findings suggest that reticulocytes and mature erythrocytes can bind specifically Epo and can respond to a rhEpo treatment. Many aspects of the reported effects of the cytokine on the circulating RBCs still remain unclear. The data we have obtained indicate that Epo regulates NO production, redox state, ion transport and cell volume, as well as glucose transport and intracellular ATP levels in mouse erythrocytes. Many of the parameters mentioned, control red cell rheology, O_2 delivery to the hypoxic tissues and erythrocyte life span. Therefore, the release of Epo into the circulation upon hypoxic stimulation has an acute protective systemic effect as it could optimize oxygen transport by already existing erythrocytes. Further studies are needed to address the systemic physiological and/or pathological consequences of Epo treatment of red blood cells in details (e.g. rheology, O_2 delivery and RBC clearance).

5.4.2 Properties of Epo binding sites

Our data revealed that reticulocytes and mature mouse erythrocytes can bind Epo specifically. The following evidence suggests that the single class of Epo binding sites that we find in mouse erythrocytes represent a classical Epo receptor: 1) pre-treatment of erythrocytes with antibodies against EpoR reduces the binding of the radiolabeled Epo to red blood cells (see section 5.3.1); 2) The dissociation constant of the receptors on mouse erythrocytes is within the range of that for the classical Epo receptor (see section 5.3.1); 3) Downstream signaling activated by Epo binding to murine erythrocytes is similar to that of a classical Epo receptor (Wojchowski *et al.*, 1999; Jelkmann *et al.*, 2008).

The inability to detect the EpoR in circulating erythrocytes reported earlier (Broudy *et al.*, 1991; Wickrema *et al.*, 1992) is most likely linked to the quality of

the antibodies against EpoR (Elliott *et al.*, 2006) and the low abundance of these receptors in the majority of cells. We have shown that Epo binding sites are not equally distributed within the RBC population and decrease drastically with erythrocyte age. Reticulocytes and young erythrocytes have a markedly higher ability to bind erythropoietin than adult and old red blood cells (young ~100; adult ~4; old ~2 Epo binding sites/cells). The marked decrease in Epo-binding capability with cell age is most likely due the release of vesicles during cell maturation (Lutz *et al.*, 1977; Snyder *et al.*, 1985). The results suggest that all erythrocytes have a potential to be Epo-sensitive, but the cellular response is most likely age-dependent.

The unequal distribution of EpoRs between the cells may have some important physiological consequences. The fast down-regulation of the red cell mass has been shown to be achieved by a selective rapid removal of reticulocytes and young cells from the population. This phenomenon known as neocytolysis is induced by reduction of the plasma Epo levels (Alfrey *et al.*, 1997; Trial *et al.*, 2004; Risso *et al.*, 2007). Neocytolysis occurs in individuals acclimatized to high altitude on descent to sea level or astronauts ascending in to space and is considered a physiological process for down-regulation of excessive red-cell mass (Alfrey *et al.*, 1997). The exact mechanism of neocytolysis is still unclear, but it was found that it is accompanied by a simultaneous decrease of Epo plasma levels and a selective removal of relatively young erythrocytes, generated over the previous 10-11 days (Rice *et al.*, 2001). Administration of exogenous Epo can rescue the young cells from a selective elimination in these cases (Rice *et al.*, 2001). It remains unclear what renders young cells more prone to clearance in the absence of Epo. The cytokine may protect erythrocytes with high numbers of receptors from being tagged to sequestration and removal, by changes in the cellular redox state, volume or metabolism (Baskurt, 1999; Lutz, 2004; Miki *et al.*, 2007). One of the possible mechanisms for the regulation of young RBC clearance is phosphatidylserine (PS) exposure. It was recently found, that young erythrocytes readily expose PS under stress conditions whereas adult and senescent cells do not (Khandelwal *et al.*, 2008). This results in a selective removal of the stressed and damaged young RBCs (Khandelwal *et al.*, 2008). The reasons for the greater sensitivity of the young erythrocytes to stress mediated PS externalization remain unclear. It is possible that the cellular machinery

responsible for PS externalization (e.g. phospholipid scramblases) is gradually lost with RBC maturation and senescence, similar to protein kinase activities (Jindal *et al.*, 1996). In addition, the higher permeability of the membranes of young cells for Ca^{2+} (Wiley *et al.*, 1977) may also contribute to the facilitated loss of the lipid bilayer PS asymmetry. The intracellular Ca^{2+} and redox-induced modification of protein-SH (sulfhydryl) groups are the main regulators of phospholipid scramblase activity (de Jong *et al.*, 2006; Sahu *et al.*, 2007). Increased cytosolic Ca^{2+} and protein oxidation enhance scramblase activity and trigger redistribution of plasma membrane phospholipids (de Jong *et al.*, 2006; Sahu *et al.*, 2007). Therefore, Epo-induced alterations in NO production and redox state of young erythrocytes would prevent the PS externalization and cellular degradation.

5.4.3 Epo-induced signaling in mouse erythrocytes

The treatment of mouse erythrocytes with Epo resulted in transient activation of PI3K/Akt pathway with RBC-eNOS being one particularly important downstream target of Akt (Mount *et al.*, 2006) present in mammalian erythrocytes (Kleinbongard *et al.*, 2006). We found that Epo triggers RBC-eNOS phosphorylation at Ser-1177 and up-regulates the enzyme activity. Our data add erythropoietin to the list of physiological regulators of the RBC-eNOS along with insulin, acting through the same signaling cascade (Kleinbongard *et al.*, 2006). Along with RBC-eNOS there might be other possible downstream targets of the PI3K/Akt pathway in the mammalian erythrocytes. One such target may be phosphodiesterase 3B, which regulates cAMP and cGMP levels in the cells (Hanada *et al.*, 2004). These two second messengers are involved in the modulation of erythrocyte ion homeostasis (Matsuura *et al.*, 1993; Petrov *et al.*, 1996; Oonishi *et al.*, 1997), cell filterability (Oonishi *et al.*, 1997; Tuvia *et al.*, 1999; Bor-Kucukatay *et al.*, 2003) and ATP secretion under shear stress or hypoxic conditions (Sprague *et al.*, 2007).

5.4.4 Nitric oxide and erythrocytes

Erythrocytes are major regulators of the circulating nitric oxide levels. Initially they were considered as a “black hole” for nitric oxide and main pathway for limiting NO bioavailability (Doyle *et al.*, 1981; Eich *et al.*, 1996). Nowadays increasing evidence indicates that RBCs are capable to control diffusion and

stability of NO in the vascular system by binding, transporting and releasing NO depending on the oxygenation of the microenvironment (Pawloski *et al.*, 2001; Muller *et al.*, 2002; Gladwin *et al.*, 2004; Kim-Shapiro *et al.*, 2006). Our data confirm the most recent findings that erythrocytes contain functionally active eNOS (Kleinbongard *et al.*, 2006) and are able to synthesize detectable amounts of NO at basal (non-stimulated) conditions, making RBCs an active player in the regulation of NO bioavailability.

We demonstrate for the first time that Epo treatment results in a time- and dose-dependent upregulation of red cell NO production. Large part of *de novo* synthesized nitric oxide is oxidized intracellularly to NO_3^- by oxy-Hb in our experimental conditions (100% oxygenation). However, significant amounts of NO escape the terminal oxidation and accumulate in the medium as NO_2^-. The role of nitrite as a potent regulator of vascular tone increased during the last years. Several studies have shown artery-to-vein gradients in plasma NO_2^- concentrations during basal conditions and during NO breathing by human subjects (Gladwin *et al.*, 2004; Kim-Shapiro *et al.*, 2006; Gladwin *et al.*, 2008). Moreover, nitrite infusion resulted in increased blood flow, accompanied by elevation in the levels of heme iron-nitrosyl complex (NO-Fe^{2+}-Hb) and *S*-nitroso-Hb (SNO-Hb). Currently it is suggested that NO_2^- can be reduced by deoxy-Hb to NO thus playing a role in hypoxic vasodilation and serving as a stable, intravascular store for NO (Gladwin *et al.*, 2004; Kim-Shapiro *et al.*, 2006; Gladwin *et al.*, 2008). In addition deoxygenation favors the release of NO bound to Hb (NO-Fe^{2+}-Hb, SNO-Hb) (Pawloski *et al.*, 2002; Kim-Shapiro *et al.*, 2006). Therefore, environmental hypoxia and the resulting increase in the circulating Epo levels would cause a significant increase in NO bioavailability by stimulating NO production from the RBCs, NO release from Hb-NO conjugates, and deoxy-Hb-catalyzed reduction of NO_2^- accumulated in the plasma to NO.

During the last years it was found that NO is crucial for the regulation of erythrocyte deformability (Starzyk *et al.*, 1999; Bor-Kucukatay *et al.*, 2003; Kleinbongard *et al.*, 2006). Previous study in our lab described several adaptive mechanisms controlling blood rheology in chronic Epo-induced erythrocytosis (tg6 mice), including elevated plasma NO levels and enhanced red cell flexibility (Vogel *et al.*, 2003). Inhibition of NO production by L-NAME application significantly impairs erythrocyte deformability and aggregability. The impaired RBC rheology

can be successfully restored by adding external NO-donors (NONOates) or L-arginine to the incubation medium (Starzyk *et al.*, 1999; Bor-Kucukatay *et al.*, 2003; Bor-Kucukatay *et al.*, 2005; Kleinbongard *et al.*, 2006). RBCs deformability is critical for their passage through the capillaries and tissue perfusion, and also determines their clearance rate. Alteration of the erythrocyte rheology can lead to their sequestration in the spleen and degradation (Baskurt, 1999). In addition, low and moderate concentration of NO donors (e.g. 0.1-1 μM NONOates) can effectively inhibit erythrocyte death induced by increased Ca^{2+} entry in to cells. The mechanism behind NO effect include independently both an increase of cGMP generation and a direct nitrosylation of the enzymes responsible for the scrambling of the cell membrane (Nicolay *et al.*, 2008). Our data imply that the Epo-induced increase in RBC NO production could be an adaptive response to maintain blood rheology, RBC survival and improving the perfusion of hypoxic tissues.

The alteration of the affinity of Hb for oxygen is another putative consequence of Epo-induced upregulation of RBC-eNOS activity. The reaction of nitric oxide with deoxy-Hb and the formation of NO-Fe^{2+}-Hb and SNO-Hb complexes is accompanied with allosterical changes of Hb that stabilize the deoxy-Hb molecule and reduce Hb-oxygen affinity (Kosaka *et al.*, 1996; Stepuro *et al.*, 2006). Thus, an augmentation of RBC-derived NO can further improve tissue oxygenation by enhancing the oxygen transfer from erythrocytes to the hypoxic areas (Kosaka *et al.*, 1996).

5.4.5 Redox-state and GSH-based signaling

The redox state is a major regulator of red cell senescence and life span (Lutz, 2004), rheological properties (Baskurt *et al.*, 2003), and oxygen carrying capacity. Our results indicate that depending on substrate availability RBC-eNOS can produce NO or $*O_2^-$ as it was reported for other cell types (Fleming *et al.*, 2003; Mount *et al.*, 2006). Epo-induced stimulation of eNOS may therefore shift the balance between the production and utilization of superoxide-derived oxidants such as hydrogen peroxide and hydroxyl radical in to a more reduced- (conditions of L-arginine saturation) or more oxidized (L-arginine deficiency) state.

On one hand, NO can display antioxidant features by serving as chain-terminating agent, thus preserving the cellular glutathione from oxidation. It reacts with different free radicals forming intermediate products that have distinct

biological activities and can be repaired by antioxidants to regenerate the original compounds (Moncada *et al.*, 1991; Pacher *et al.*, 2007). On the other hand, superoxide anions react very fast with Fe^{2+} iron, generating hydrogen peroxide and the highly reactive hydroxyl radicals (Frein *et al.*, 2005), leading to oxidative damage of cellular components. Alternatively, under some conditions eNOS can produce simultaneously NO and $*O_2^-$, forming peroxynitrite ($ONOO^-$) (Pacher *et al.*, 2007). It was found that low levels of peroxynitrite can exert cytoprotective properties by activating pentose phosphate pathway, increasing NADPH levels and improving GSH regeneration (Garcia-Nogales *et al.*, 2003; Bolanos, 2004). Therefore, Epo-induced up-regulation of RBC-eNOS activity has a direct effect on the cellular redox state and the antioxidant capacity of the RBCs.

We found that in the presence of L-Arg, application of therapeutic doses of rhEpo (100 U/ml) to RBCs for 30 - 60 min increased the levels of GSH and decreased GSSG cellular content. The Epo-induced change in the GSSG/2GSH ratio shifted the half-cell redox potential to more reduced state, making the cells more resistant to oxidative stress. Similar changes in erythrocyte glutathione levels were observed in rats undergoing heterotopic heart transplantation, treated with 5000 U/kg rhEpo (see section 6.3.3). It is possible that lower doses of the cytokine may also shift the cellular redox state when present for longer time periods. Currently, the mechanism of the GSSG/2GSH ratio changes induced by NO is unclear.

Glutathione controls the levels of free radicals by being a direct free radical scavenger and providing the reducing capacity for glutathione peroxidases and glutathione *S*-transferases that play an important role in detoxification of hydrogen peroxide, other peroxides and free radicals (Hayes *et al.*, 1999; Pastore *et al.*, 2003; Sharma *et al.*, 2004). GSH also participates in trans-hydrogenation reactions (S-glutathionylation) forming reversible adducts (glutathionylated thiols) protecting different proteins against oxidation of critical cysteine residues including glutathione S-transferase, superoxide dismutase, hemoglobin, cytoskeletal and extracellular proteins (Klatt *et al.*, 2000; Pastore *et al.*, 2003). It was recently reported that S-glutathionylation is involved in the control of the activity of specific enzymes, as protein tyrosine phosphatases (Peter Klatt, 2000; Pastore *et al.*, 2003).

Epo-induced effects on the cellular redox state have particularly important consequence for the rhEpo therapy of patients with kidney failure. Increased oxidative stress is described in patients with chronic renal failure undergoing hemodialysis (HD) (Jacob *et al.*, 1975; Loughrey *et al.*, 1994; Nguyen-Khoa *et al.*, 2001). Several authors reported that a rhEpo-treatment of uremic patients on chronic hemodialysis is accompanied by further increase of oxidative stress and requires co-application of antioxidants (Linde *et al.*, 1992; Cristol *et al.*, 1997; Nemeth *et al.*, 2000). Our experiments revealed that in the absence of L-Arg, even low levels of Epo (1 U/ml) are sufficient to cause a significant glutathione oxidation, most probably due to an increased $*O_2^-$ formation by RBC-eNOS. Furthermore, it was shown that in different pathological conditions L-Arg uptake is inhibited (Maarsingh *et al.*, 2004; Brunini *et al.*, 2005; Yang *et al.*, 2006) and extracellular L-Arg content is altered (Wu *et al.*, 1999; Brunini *et al.*, 2005; Brunini *et al.*, 2006) leading to impairment of the NO production. Our data suggest that the deleterious effects of Epo therapy might be due to impaired eNOS activity and increased $*O_2^-$ generation instead of NO. Therefore further investigations of the function of RBC-eNOS in pathology are required for improving the erythropoietin treatment and to gain maximal positive- and omit the possible negative side effects of the cytokine therapy.

The Redox state is an important factor controlling the cell fate and survival (Schafer *et al.*, 2001 169). Erythrocytes are continuously exposed to oxidative stress in the circulation, and undergo various oxidative damages. These modifications include formation of methemoglobin, increased lipid peroxidation, oxidative modifications and degradations of proteins, cross-linking of integral membrane proteins, attachment of hemoglobin to membrane cytoskeletal proteins (mostly to spectrin), altered passive cation permeability and surface properties (Baskurt *et al.*, 2003). It was shown that band 3 molecules (anion transport protein) on the membrane tend to form clusters upon RBC oxidation (Lutz *et al.*, 1987; Lutz *et al.*, 1988). The oligomers of band 3 are a prerequisite for anti-band 3 naturally occurring antibodies (NAb) binding to human erythrocytes facilitating RBC clearance by the phagocytes (Lutz *et al.*, 1988; Lutz, 2004). Therefore the effects of Epo on RBCs redox state may affect the structural and functional integrity of the erythrocytes and their survival.

5.4.6 Epo and ion transport

Our experiments revealed that Epo regulates the activity of the volume-defining ion transporters responsible for maintaining cell deformability. We found that the treatment of mouse erythrocytes with rhEpo stimulates potassium uptake through the Na^+/K^+-pump with a concomitant inhibition of ion leakage through NKCC. Together, these changes result in alteration in ion/water balance and increased cell volume.

The regulation of the Na^+/K^+-ATPase activity is a complex, tissue-specific process involving either protein phosphorylation (Therien *et al.*, 2000) mediated by a variety of kinases (PKA, PKG, PKC, tyrosine kinases) and phosphatases (PP1 and PP2B), or redox modulation of critical cysteine residues (Bogdanova *et al.*, 2006). The major signal transduction mechanism responsible for the physiological effects of NO is the activation of soluble guanylate cyclase and the increase of cGMP levels in the cells (Pacher *et al.*, 2007). cGMP is known to be involved in the regulation of Na^+/K^+-pump (de Oliveira Elias *et al.*, 1999). Therefore, it is possible that the Na^+/K^+-pump is either a direct downstream target of Epo signaling in mouse RBCs, or the regulation of its activity is secondary to the Epo-induced alteration of the cellular redox state or activation of RBC-NO production. The mechanisms behind the regulation of NKCC activity are much less defined. It is known that phosphorylation and interaction with F-actin are the main activators of NKCC activity, however the responsible phosphatase and kinases are currently unknown (Flatman, 2002). Our preliminary experiments revealed that Epo does not affect Na^+/K^+-ATPase and NKCC activity in erythrocytes from eNOS deficient mice (data not shown). Based on this data we can hypothesize that ion transporters are not direct targets of Epo-sensitive signaling cascades, but the regulation of their activity is secondary to the Epo-induced changes in erythrocyte NO production or redox state.

The Na^+/K^+-ATPase and NKCC are involved in the regulation and maintenance of volume homeostasis in the cells (Cossins *et al.*, 1997; Lambert *et al.*, 2008). Among the numerous transformations that take place during the differentiation and maturation of erythroid cells, cell volume regulation represents one of the well-recognized cellular events (Gallicchio *et al.*, 1979; Delpire *et al.*, 1994; Pellegrino *et al.*, 1998). Increased potassium content and cell volume are essential promoters for the normal erythroid progenitor growth between BFU-E &

and CFU-E stages (Gallicchio *et al.*, 1979). Interestingly, EpoR expression starts at BFU-E and peaks in CFU-E stage (Fisher, 2003), where the cell volume changes of erythroid precursors are maximal. During the late stages of hematopoiesis there is a marked reduction in the inorganic ion co-transport systems, correlating with the observed cell volume reduction and decrease in EpoR numbers (Delpire *et al.*, 1994; Pellegrino *et al.*, 1998). Based on Epo-induced effects on ion transport in mature erythrocytes, we hypothesize that the volume alterations during RBC development are triggered by Epo and depend on the expression level of EpoR in the erythroid progenitors.

Two important properties of mature erythrocytes are regulated by the changes of RBCs volume, namely cellular deformability and clearance. RBC deformability is one of the major determinants of blood fluidity and tissue perfusion (Baskurt *et al.*, 2003). It is affected by alterations of the properties and associations of cytoskeletal proteins, the ratio of RBC membrane surface area to cell volume, cell morphology, and cytoplasmic viscosity (Baskurt *et al.*, 2003). The degree of hydration of the cells determines hemoglobin concentration, cytoplasmic viscosity, and the surface area/volume relationship. Therefore, the Epo-induced regulation of the ion/water content could be beneficial for maintaining the normal cytoplasmic viscosity and erythrocyte deformability. Cell deformability is crucial for the RBCs to pass through the spleen and thereby determines the rate of their degradation (Baskurt, 1999). Our data imply that along with *de novo* RBC production, Epo might also regulate erythrocyte survival by modulating ion/water content and cell volume.

5.4.7 Epo and RBC metabolism

Finally, our results confirmed previous reports on Epo-induced regulation of sugar transport, across the erythrocyte membranes (Baciu *et al.*, 1983; Ghosal *et al.*, 1987). Nevertheless, the mechanism by which Epo affects glucose uptake remains unclear. Since reticulocytes and mature erythrocytes no longer possess machinery for *de novo* protein synthesis and vesicular trafficking, we can exclude increased glucose transporters (GLUTs) expression and enhanced intracellular trafficking of GLUTs from the putative mechanisms mediating Epo action (Behrooz *et al.*, 1999). It was shown that GLUTs activity is upregulated by PI3K- (Gould *et al.*, 1994) and cAMP-mediated signaling (Kashiwagi *et al.*, 1983). As previously

discussed, these pathways are present in RBCs (Minetti *et al.*, 1997) and are Epo-sensitive, making PI3K or cAMP suitable candidates for mediators involved in Epo-induced regulation of glucose transport in the erythrocytes. Another possible mechanism includes eNOS and $ONOO^-$ production. Peroxynitrite stimulates tyrosine-dependent signal transduction via a reversible inhibition of phosphotyrosine phosphatase. This leads to enhanced band 3 tyrosine phosphorylation, a translocation of glycolytic enzymes from the band 3 to the cytoplasm, and as a consequence to an increased glucose metabolism (Mallozzi *et al.*, 1997). Furthermore, it was reported that NO donors (e.g. NONOates) can increase the rate of glycolysis and energy production in rat erythrocytes without any change in the cellular ATP levels, suggesting the concomitant stimulation of the ATP-consuming processes (Maletic *et al.*, 2000). In our experiments Epo also activated glucose uptake and evidently the metabolic rate, without significant alterations of the cellular ATP content. Epo-induced upregulation of Na^+/K^+-pump activity and respectively increase in ATP consumption is a possible explanation for the obtained results (see chapter 5.3.6).

Glucose is the main source of energy and redox equivalents (NADPH) in the RBCs. Moreover, the intracellular levels of the intermediate glycolytic product 2,3-bisphosphoglycerate directly regulate Hb-oxygen affinity (Brewer, 1974). 2,3-DPG plays an important role in the adaptation of the organisms to hypoxic environment, since its accumulation in the RBCs enhances the release of oxygen from erythrocytes, to hypoxic tissues (Brewer, 1974). It was reported that Epo administration in healthy humans elevates the 2,3-DPG levels independently form the reticulocyte number alterations triggered by the cytokine (Birgegard *et al.*, 2001). Epo-induced increase of glucose uptake in mature erythrocytes followed by elevation of the glycolytic rate would be a possible explanation for the cytokine effects on 2,3-DPG levels.

5.5 Conclusions and Outlook I

The data we have obtained indicate that Epo mediates an acute, adaptive response in mature mammalian erythrocytes by a rapid stimulatory effect on RBC-eNOS. This results in a shift of the cellular redox state to a more reduced or more oxidized state depending on the L-Arg availability. We also demonstrate that Epo-treatment of mouse erythrocytes may regulate the activity of the ion and glucose transporters. Further work is needed to establish the mechanisms of the observed effects of Epo on the erythrocytes and the different downstream targets of the Epo-sensitive signaling cascades. In particular, the role of NO in Epo-induced regulation of cell volume, glucose uptake remains to be clarified.

Several studies have indicated that Epo is involved in the regulation of oxygen caring capacity of RBCs (Birgegard *et al.*, 2001), blood viscosity (Vogel *et al.*, 2003) and erythrocyte survival (Myssina *et al.*, 2003). We would like to investigate whether Epo-induced responses that we have observed in erythrocytes affect the affinity of Hb for oxygen, RBC deformability and life span.

We would like to use the obtained data for optimization of the Epo treatment protocols in human patients to avoid side effect such as increased oxidative stress (Linde *et al.*, 1992; Cristol *et al.*, 1997; Nemeth *et al.*, 2000). Finally, we would like to assess the role of Epo in the acute regulation of red cell mass, unraveling the mechanisms of neocytolysis.

In conclusion, this work is a first step in characterizing the Epo effects on mature red blood cells. Many more steps are needed to understand the mechanisms of acute Epo effects and the physiological role of Epo and its receptors in circulating erythrocytes. Our findings indicate that Epo exerts a wide range of effects in mature erythrocytes that could affect their survival and functional properties as oxygen carriers. Therefore along with the Epo-triggered increase in the red cell number, the cytokine-induced responses in already existing RBCs might represent an acute, adaptive mechanism to improve tissue oxygenation and to cope with the hypoxic challenge.

6. CHARACTERISATION OF THE CARDIOPROTECTIVE PROPERTIES OF ERYTHROPOIETIN

6.1 *Erythropoietin + heart = cardioprotection…* but how?

Ischemia-reperfusion (I-R) injury is the major cause of myocardial cell death and the high morbidity and mortality rates during open heart surgery, trauma, cardiovascular disease and transplantation (Murphy *et al.*, 2008). I-R injury is defined as myocardial loss caused by an ischemic period, followed by restoring of perfusion (Buja, 2005; Yellon *et al.*, 2007; Murphy *et al.*, 2008). Extensive research has focused on developing different strategies for cardioprotection and improving myocardial resistance to I-R injury (Yellon *et al.*, 2007; Murphy *et al.*, 2008). During the last years numerous studies suggested that Epo as pleiotropic, cytoprotective factor could be beneficial in the treatment of myocardial infarction and the concomitant I-R (Jelkmann, 2007; Arcasoy, 2008). However, despite the exponential growth in the number of publications on the cardioprotective effects of Epo, its targets in the myocardium and the molecular mechanisms behind its cardioprotective effects remain largely unclear.

6.1.1 Presence of erythropoietin receptors in cardiomyocytes

Currently, the reports on the presence of EpoR on adult cardiomyocytes are controversial. It was found that EpoR plays an important role in heart development, promoting proliferation of cardiac myocytes (Wald *et al.*, 1996; Ogilvie *et al.*, 2000). Expression of the EpoR in embryonic heart has been proven by using in situ hybridization technique (Wu *et al.*, 1999). The highest density of the receptor is found in epicardium and not in cardiac myocytes (Wu *et al.*, 1999; Stuckmann *et al.*, 2003). The stimulation of cardiomyocyte proliferation by Epo during the embryonic development is initiated by factors secreted by the epicardium upon the binding of the cytokine to its receptor. Other studies reported the presence of EpoR protein in cardiomyocytes of adult mammalian heart (Tramontano *et al.*, 2003; Wright *et al.*, 2004; Depping *et al.*, 2005). However, these findings were questioned because the antibodies used for detection by means of immunofluorescence and immunohistochemistry were later proven to give false-positive staining in Epo-knockout tissue (Elliott *et al.*, 2006). Additionally, it was shown that Epo-induced cardioprotection is absent in knockout mice lacking

the common β-receptor (βcR) subunit (Brines *et al.*, 2004). This data raised the possibility of the existence of a nonerythroid, cytoprotective, heteromeric erythropoietin receptor, containing common EpoR subunit and other membrane molecules, such as βcR.

6.1.2 Epo-induced cardioprotection – *in vitro* studies

Several studies investigating the cardioprotective effect of Epo used cardiomyocytes isolated from adult rat hearts, maintained in culture. The cells were subjected to sustained hypoxia or oxidative stress. Administration of rhEpo to the culture medium prior to the stress stimulus reduced the rate of apoptosis by 50% (Calvillo *et al.*, 2003; Parsa *et al.*, 2003; Tramontano *et al.*, 2003). In addition, it was demonstrated that the protective action of rhEpo requires the activation of PI3K/Akt pathway, because the PI3K inhibitor wortmannin abolished rhEpo's favorable action on cardiomyocytes. However, the downstream targets of the acute Epo-induced signal transduction in the cardiac cells remain poorly characterized.

6.1.3 Epo-induced cardioprotection – *ex vivo* and *in vivo* studies

Ex vivo experiments, using Langendorf isolated rodent heart preparations exposed to warm ischemia-reperfusion, showed that Epo improves the functional recovery of the left ventricular developed pressure, coronary flow and cellular damage during the reperfusion phase (Cai *et al.*, 2003; Cai *et al.*, 2004; Shi *et al.*, 2004; van der Meer *et al.*, 2004; Wright *et al.*, 2004; Bullard *et al.*, 2005; Hanlon *et al.*, 2005; Rafiee *et al.*, 2005). Furthermore, *in vivo* studies using left coronary ligation model of myocardial infarction, revealed that Epo administration results in a reduction of the infarct size, improvement of cardiac contractility and normalization of hemodynamic function (Calvillo *et al.*, 2003; Moon *et al.*, 2003; Parsa *et al.*, 2003; Tramontano *et al.*, 2003; Bullard *et al.*, 2005; Hirata *et al.*, 2005; Lipsic *et al.*, 2006). It was shown that Epo-induced cardioprotection at least partially is due to suppression of apoptosis and improvement of cell survival via activation of several antiapoptotic and mitogenic signaling cascades, including PKC_{ε}, MAPK/Erk, PI3K/Akt and STAT-3 (Cai *et al.*, 2004; Shi *et al.*, 2004; Bullard *et al.*, 2005; Hanlon *et al.*, 2005; Hirata *et al.*, 2005; Rafiee *et al.*, 2005; Nishihara *et al.*, 2006).

Most of the above presented studies use a coronary occlusion model of acute myocardial infarction (MI) (Moon *et al.*, 2003; Tramontano *et al.*, 2003; Bullard *et al.*, 2005; Lipsic *et al.*, 2006). The narrow time window of cardioprotection for rhEpo reduces significantly its therapeutic potential when treating MI (first hours after the injury) (Moon *et al.*, 2005; Arcasoy, 2008). Open-heart surgical procedures (e.g. coronary artery bypass, valve replacement and heart transplantation) represent on one hand additional stress to the heart, subjected to global I-R. On the other, the pre-operative period allows a precisely timed administration of the drug allowing maximal cardioprotective effect of rhEpo. The heterotopic heart transplantation in rats represents a suitable *in vivo* model of cold, global ischemia and warm reperfusion, mimicking closely the clinical settings during open-heart surgery (Galinanes *et al.*, 1991; Wheatley, 2002). However, until now there were no studies using this model to investigate the possible cardioprotective effects of Epo.

Despite the rapidly growing numbers of papers, the question about the primary cellular Epo targets, when the cytokine is administrated iv is poorly addressed. As discussed in chapter 6.1.1 the presence of EpoR in the adult, mammalian cardiomyocytes is questionable and it was shown that physiologically relevant, Epo-induced responses are at least partially mediated by the epicardium (Wu *et al.*, 1999; Stuckmann *et al.*, 2003). Furthermore, erythropoietin is a relatively large glycoprotein (see section 4.1.1) and it is very unlikely that it can pass freely through the blood vessel wall, unless the latter is severely damaged. Up to now, there is no evidence for active trafficking of Epo by the endothelium. All these facts suggest that *in vivo,* cardioprotective effects of Epo are most likely not mediated by reduction of apoptosis as shown in isolated cardiomyocyte preparations. It is more likely, that when in the circulation, Epo interacts with blood vessel endothelium triggering the release of paracrine factors responsible for the observed effects of the cytokine on the myocardium. A suitable candidate for such paracrine messenger is nitric oxide. So far, a direct involvement of NO in Epo-induced suppression of apoptosis in the heart was reported in a single publication (Burger *et al.*, 2006). In this study, using neonatal cardiomyocyte culture and *in vivo* experiments, norepinephrine- or hypoxia-induced apoptosis was attenuated by Epo, via an increase in eNOS expression, phosphorylation and NO production. Blockage of eNOS function by L-NAME diminished the Epo-induced

cardioprotection. In accordance to these results, Epo showed limited cardioprotection in eNOS knockout animals (Burger *et al.*, 2006).

6.2 General objectives

The aim of this ongoing study is to characterize the *in vivo* and *in vitro* cardioprotective properties of rhEpo, using a heterotopic rat heart transplantation model of cold global I-R injury and primary neonatal rat cardiomyocyte cultures. We further investigate the putative mechanisms behind rhEpo-induced responses in the heart by:

- characterization of the pharmacokinetics and dynamics of Epo interaction with the heart
- determination of the primary cellular targets of Epo, *in vivo* and *in vitro*
- further investigation of the possible treatment strategies of rhEpo against cold, global ischemia-reperfusion injury
- elucidating the putative mechanisms of an Epo induced acute cardioprotection by comparing its role as anti-apoptotic and anti-necrotic factor in our *in vivo* experimental model of cold global ischemia-reperfusion injury

In heterotopic heart transplantation experiments we used male Lewis rats (250 - 300 g) as donors and recipients. The hearts of donor animals (grafts) were arrested with cold crystalloid cardioplegia and stored in the same cardioplegic solution at 4°C until implantation (Fig. 14). The grafts were transplanted heterotopically in the abdomen of recipients, according to the technique of Ono and Lindsey (Ono et al., 1969). The total duration of global ischemia was 45 min of which the last 15 min were needed for anastomoses. The ischemia period was suggested by the clinicians as an average time for cardiac arrest during coronary bypass operation.

Recipient rats were randomized to control (non-treated) or Epo-treated group receiving 5000 U/kg of rhEpo intravenously 20 min prior to reperfusion (Fig. 14). The dose and administration protocol used in our study has been shown previously to confer significant cardioprotection against I-R injury (Hirata et al., 2005; Moon et al., 2005). At 5 time points (5, 30, 60, 360 and 1440 min) after the onset of reperfusion, the recipients were sacrificed. Blood, native and grafted hearts were harvested for subsequent analyses (Fig. 14). During the first 24 h after the operation, cardiac surgery patients are at highest risk, therefore we investigated Epo-induced cardioprotection within this time period.

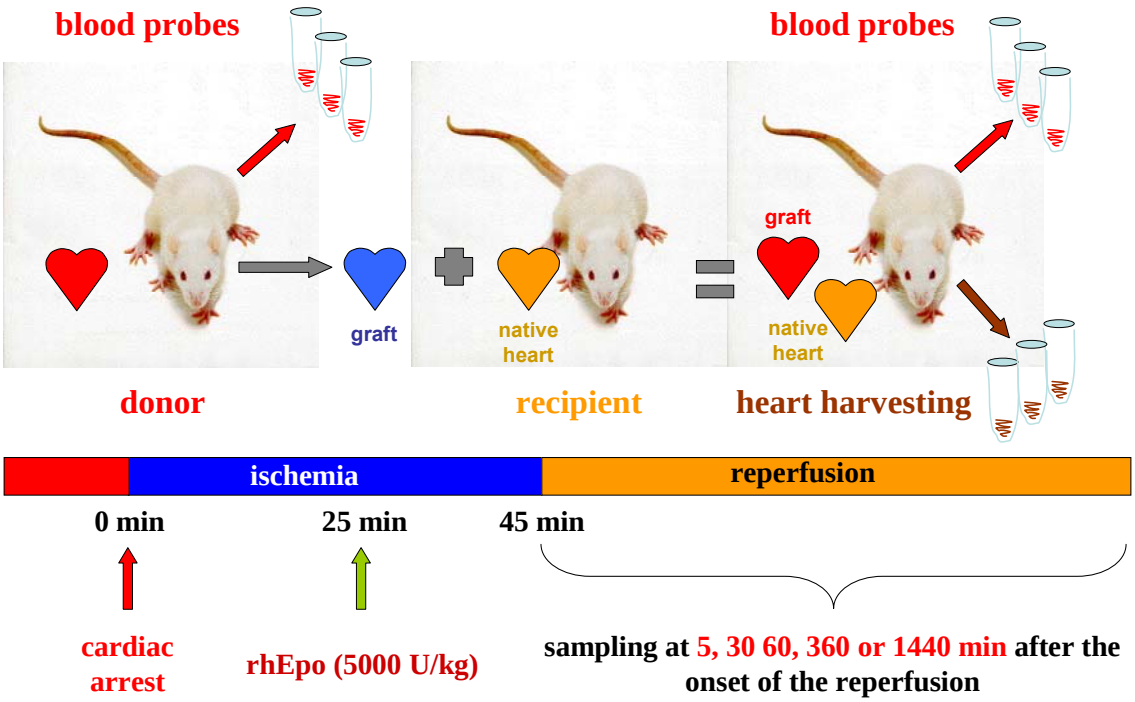


Figure 14. Scheme of heterotopic heart transplantation experimental protocol.

In our experimental model rhEpo comes in contact with the grafted heart upon the restoration of reperfusion. This means that the putative cardioprotective effects of Epo will not be due to suppression of ischemic injury of the heart but rather to decreasing of reperfusion-induced damage.

Neonatal rat cardiomyocytes (NRCs) for the *in vitro* studies were isolated from Wistar rat pups of postnatal days 3-4 (Schafer et al., 1988). The cells were used for experiments within 5 days after isolation.

6.3 Own research (based on Papers 3 and 4)

6.3.1 Pharmacokinetics and Epo binding in the heart

Our results revealed that heterotopic rat heart transplantation resulted in reduction in basal (endogenous) Epo plasma levels (86.1 ± 2.3 mU/ml) by 40% (Paper 3). The cytokine plasma store remained depleted for the following 24 h after the surgical intervention. An iv application of 5000 U/kg rhEpo to the recipient animals 20 min prior to the onset of reperfusion, increased the cytokine levels in the blood stream up to 130 ± 7 U/ml immediately after the administration. The half life of rhEpo in rat plasma was about 4.9 h with the cytokine levels still exceeding the basal ones 24 h after the administration (Paper 3).

The endogenous Epo content in homogenates of native hearts of recipients was low (0.12 ± 0.04 mU/mg protein) and remained unaltered in both native and transplanted myocardium of the control animal group over the 24 h of reperfusion (Paper 3). Intravenous administration of hrEpo caused a rapid increase in hrEpo in both native and transplanted heart already within the first minutes of exposure. The acute Epo binding capacity did not differ for the native and transplanted hearts, but the clearance rate was 8-fold higher in the grafts compared to the native heart ($T_{1/2}$ of ~1.8 h in transplanted and that of ~15.2 h in native hearts, Paper 3).

To evaluate the primary localization of rhEpo within the myocardial tissue, we have stained cryosections of the cytokine-treated and control (saline-treated) hearts using highly specific anti-Epo antibodies (Papers 3 and 4). A strong and specific staining was observed only in the vascular endothelium in Epo-treated hearts (native hearts and grafts) in comparison to control preparations (Papers 3 and 4). We were unable to detect hrEpo binding to the cardiac myocytes at any time point between 5 min and 24 h of perfusion. Interestingly, a weak Epo staining was observed in the coronary endothelium of the grafted hearts but, not the native ones 5 min after the onset of perfusion (Paper 3). This observation was in line with the decrease in plasma endogenous Epo levels in the recipient animals.

6.3.2 Epo binding and intracellular processing in NRCs

To test if cardiomyocytes can directly interact with erythropoietin we used primary culture of neonatal rat cardiomyocytes (NRCs). Epo binding was assessed

using ^{125}I-hrEpo as a marker as well as by means of immunohistochemistry. Our findings revealed that rat cardiomyocytes can specifically bind Epo (Paper 3). The cytokine remained membrane-bound for the first 15 min with subsequent internalization, sequestration in cistern-like compartments and degradation occurring within 30-60 min (Paper 3). After 3 h of treatment cells were desensitized to external hrEpo. The bound hrEpo pool was degraded and de novo binding did not occur despite the continuous presence of hrEpo in the cell culture medium.

6.3.3 Epo protects the heart against cold, global I-R injury

I-R following heterotopic heart transplantation resulted in an increase of the plasma cardiac troponin-T (TnT) levels, indicating pronounced and acute cardiomyocyte damage (Paper 4). The mechanical overload of the heart upon the restoration of reperfusion was manifested by a significantly elevated atrial- (ANP) and brain- (BNP) natriuretic peptides levels into the circulation. Application of 5000 U/kg rhEpo prior to reperfusion, decreased TnT, ANP and BNP plasma levels (Paper 4). In addition, the recovery of the contractile activity after the restoration of blood flow was faster in rhEpo-treated hearts in comparison to non-treated controls. Six hours after the onset of perfusion an increase in plasma IL-6 level was observed in both Epo-treated and control animals, indicating a systemic inflammatory response. Our data revealed that IL-6 plasma levels were not affected by hrEpo administration (Paper 4). Thus, the cardioprotective effect of Epo was not due to suppression of the secondary inflammatory response.

6.3.4 Mechanisms of Epo-induced cardioprotection – the role of apoptosis

To evaluate the role of apoptosis in I-R injury caused by heterotopic heart transplantation and its possible sensitivity to rhEpo treatment, we monitored several apoptotic markers in transplanted and non-transplanted (native) hearts (Paper 4). These include activity of caspases 3 and 9, PARP cleavage and TUNEL staining.

The basal activity of caspase 3 and 9 in the native heart tissue was very low in comparison to staurosporine-treated vascular endothelial cells used as positive control (0.28% of the positive control for caspase 3 and 0.20 % of the positive control for caspase 9). I-R resulted in a very modest increase in the activity of both

caspases which was only statistically significant between the native and the transplanted hearts 30 min after the onset of perfusion. The rhEpo treatment had no statistically significant effect on caspases activity (Paper 4).

In accordance with the low caspase activity we did not detect significant PARP cleavage in transplanted hearts at any reperfusion time point. In addition, the number of TUNEL-positive cells ranged between 0 and 4 cells/field (magnification 20x) and did not differ between non treated and rhEpo treated ischemic hearts. Taken together with the data on the release of cardiac TnT into the circulation these data suggest that most of the ischemia-reperfusion damage represents oncosis/necrosis rather than apoptosis.

6.3.5 Mechanisms of Epo-induced cardioprotection - necrosis

The main hallmarks of necrosis, along with the release of intracellular components (e.g. troponin-T) into the blood stream are cell swelling and increased oxidative stress.

I-R triggered by the heterotopic heart transplantation was followed by a transient increase of tissue sodium and water levels, developing in the grafts during the ischemic phase and ameliorating during the first minutes of reperfusion in the control recipient group. rhEpo treatment facilitated the recovery of the grafts from ischemia-induced tissue swelling (Paper 4).

Oxidation is one of the recognised causes of myocardial damage at reperfusion. Indeed, reperfusion resulted in acute depletion of the GSH pool in grafted ventricular tissue, which became even more pronounced with time (Paper 4). Marked reduction of GSH levels was observed also in erythrocytes and plasma, suggesting systemic increase in oxidative stress. Transplanted hearts of the hrEpo-treated group did not show GSH depletion at any reperfusion time point (Paper 4). Furthermore, the administration of rhEpo resulted in an increase in the GSH content in native myocardium in comparison to non-treated controls. In addition, hrEpo was able to abolish transplantation-induced reduction of the GSH levels in erythrocytes and plasma of the recipient animals, thus providing systemic defence from the reperfusion-triggered oxidative stress (Paper 4).

6.3.6 Downstream targets of hrEpo in the heart tissue and cardiac cells

Intravenous administration of rhEpo did not cause any detectable increase in phosphorylated Akt levels in ventricular tissue homogenate (Paper 3). However the immunohistochemical identification of the phosphorylated Akt in cryosections showed a transient up-regulation of Akt phosphorylation solely in coronary endothelium of the grafts and native hearts. The activation of Akt could only be observed in the tissue exposed to hrEpo for 5-30 min (Paper 3). Our further experiments revealed that PI3K/Akt pathway in the cardiomyocytes was also Epo-sensitive. The treatment of NRCs with 10 U/ml of hrEpo resulted in transient phosphorylation of Akt, appearing after 30 min of incubation (Paper 3).

As mentioned previously (see section 6.1.3) nitric oxide is a known downstream target of the PI3K/Akt pathway and a suitable candidate as a paracrine mediator of Epo-induced cardioprotection. Therefore, we stained heart cryosections with an antibody against eNOS phosphorylated at serine-1177 residue (active form of the enzyme). rhEpo treatment resulted in an increase in eNOS phosphorylation in both native and grafted hearts 5, 30 and 60 min after the onset of reperfusion (Papers 3 and 4). Cardiac myocytes are known to express eNOS along with vascular endothelial cells. hrEpo iv administration resulted in activation of eNOS selectively in vascular endothelium of coronary vessels but not in the myocardial tissue itself (Papers 3 and 4). However, when cardiomyocytes (NRCs) are directly exposed to rhEpo, phosphorylation of eNOS at Ser-1177 is also observed (Paper 3). 6 and 24 h after the heterotopic heart transplantation a strong increase in phosphorylation of eNOS at Ser-1177 in blood vessel walls of control hearts (native and transplanted) was observed, corresponding with the manifestation of a systemic inflammatory response. At these late time points (6 and 24 h after the onset of reperfusion) the cytokine-treated animals showed significantly lower eNOS activation in comparison to control ones (Paper 4).

Simultaneously with eNOS phosphorylation, plasma nitrite levels in the rhEpo-treated recipient animals were significantly elevated in comparison to control animals (saline-treated) at the early time points (5, 30 and 60 min after the onset of reperfusion), indicating an increase in nitric oxide production (Paper 4). Furthermore, in accordance with the changes in eNOS phosphorylation status 6 and 24 h after the transplantation, rhEpo administration was accompanied with reduced NO_2^- plasma levels at these time points in comparison to saline-treated controls (Paper 4).

Up-regulation of the nitrite/nitrate production was observed also in the medium of NRCs treated with rhEpo (Paper 3). In addition, the cytokine administration in the cell-culture medium, resulted in stimulation of the active influx of K^+ mediated by the Na^+/K^+-ATPase along with suppression of passive K^+ movement across the sarcolemma (Paper 4). This did not occur when the incubation medium was deprived of L-Arg, suggesting that the observed effects of hrEpo on both the Na^+/K^+-ATPase and the passive K^+ flux are not direct but secondary to the Epo-induced stimulation of NO production (Paper 4). Interestingly, similar effect of Epo on ion transport was observed in mouse erythrocytes (see section 5.3.6).

6.4 Discussion II

6.4.1 Major findings

In line with the reports of other groups (Bogoyevitch, 2004; Hanlon *et al.*, 2005; Rafiee *et al.*, 2005; Maiese *et al.*, 2008) we demonstrate that Epo is a potent cardioprotective factor. We showed for the first time that iv administration of rhEpo prior to reperfusion significantly reduces myocardial damage triggered by cold, global, ischemia-reperfusion injury. Based on the obtained results, we can emphasize two important findings concerning Epo-induced cardioprotection:

1) Erythropoietin, secreted or injected into the blood stream, is not able to pass through the blood vessel wall. The primary *in vivo* cellular targets of the cytokine are the vascular endothelial cells. Epo-induced cardioprotection *in vivo* is most probably mediated by endothelium-derived factors, one of which is nitric oxide.

2) Neonatal cardiomyocytes can bind Epo specifically and respond to the cytokine treatment. Epo administration to the NRCs leads to activation of the PI3K/Akt pathway, followed by eNOS upregulation, as observed previously for endothelial cells and erythrocytes.

6.4.2 Cellular targets of Epo in the heart tissue

Our results show for the first time that iv administered rhEpo does not pass through the blood vessel wall, but reacts predominantly with the endothelial cells. The Epo binding capacity decreased with time, most probably because of the internalization and degradation of the receptor-enzyme complex. The binding of Epo to the endothelial cells is a key determinant of its plasma pharmacokinetics. In the plasma of young and healthy rats $T_{1/2}$ for the hrEpo depends on the administered dose ranging from 4.8 to 8.8 h (Woo *et al.*, 2007) and is similar to the data obtained in humans (4-9 h, (Fisher, 2003)). The results we have obtained ($T_{1/2}$ of 4.87 h) were within this range, although Epo clearance in our experimental model somewhat exceeded that in healthy animals for the corresponding dose (Woo *et al.*, 2007). This suggests that stress during the transplantation facilitates Epo clearance and degradation. In support of this hypothesis, we observed an impressive acceleration in the processing of the hrEpo bound in the transplanted hearts as compared to the native hearts (Paper 3). Moreover, a weak Epo staining

can be detected in transplanted control hearts, but not in the corresponding native ones 5 min after the onset of reperfusion. This observation is in line with a decrease in endogenous Epo plasma levels of the recipient animals. Our data imply that stress could induce either recruitment of additional Epo binding sites or an increase in the affinity of already available ones. The latter is more likely as the total amount of rhEpo detected in transplanted hearts was not different from that in native hearts, indicating that in the presence of excessive amounts of Epo, the binding capacity of the graft and native heart is the same (Paper 4).

The kinetics of Epo interaction with EpoR in the epithelial cells is currently under investigation. However, activation of Epo-sensitive signaling in HUVECs was reported within 30 min after the cytokine administration (Beleslin-Cokic *et al.*, 2004). In erythroid precursor cells interaction of Epo with its receptor occurs within minutes reaching maximum after 20 min of treatment with the cytokine (Gross *et al.*, 2006). Most of the Epo-EpoR complexes are quickly internalized and degraded in the proteasomes and lysosomes, limiting the duration of Epo-induced signaling (Walrafen *et al.*, 2005; Gross *et al.*, 2006). In agreement with these results, the Epo-binding capacity of the coronary endothelium was transient, despite the persistently high cytokine plasma levels within 24 hours after its administration (Paper 3 and 4).

The *in vitro* studies we performed showed undoubtedly that neonatal cardiomyocytes have Epo binding sites and respond to the cytokine treatment (Paper 3). The properties of these binding sites in NRCs resemble those in erythroid cells, including binding and degradation kinetics, and PI3K/Akt pathway activation. However, a detailed investigation of the nature of these binding sites is still in progress. The presence of Epo binding sites in neonatal cardiomyocytes most likely supports the proliferation of the embryonic myoblasts by Epo released from the epicardium in the embryonic heart (Wald *et al.*, 1996; Stuckmann *et al.*, 2003). Based on the present findings we cannot confirm or disapprove the ability of adult cardiomyocytes to bind Epo *in vivo* as they remain inaccessible to the intravenously administered cytokine. Several reports suggested that adult cells possess EpoR identical to that present in the hematopoietic cells (Calvillo *et al.*, 2003; Wright *et al.*, 2004; Mao *et al.*, 2008). However, no attempts were so far made to characterize the Epo binding proteins in details, e.g. by means of mass spectrometry, and the specificity of the antibodies used for detection of EpoR was

questioned (Elliott *et al.*, 2006). If adult cardiomyocytes preserve their Epo-binding capability, it remains unclear whether locally produced Epo can exert significant cytoprotective properties, as the myocardium remains inaccessible to the blood-born cytokine. In addition, up to now there is no data reported on Epo expression by cardiomyocytes or myocardial fibroblasts.

6.4.3 Mechanism of Epo-induced cardioprotection – apoptosis vs. necrosis

Myocardial I-R injury is the major cause of myocardial cell death in several pathological conditions, including stenosis of coronary vessels, myocardial infarction, open heart surgery and transplantation (Murphy *et al.*, 2008). Several processes have been implied to be the cause of I-R-induced myocardial damage of which calcium-overload, inflammation and oxidative stress are the major ones (Yellon *et al.*, 2007). We have observed all these characteristic signs of I-R injury in our experimental model. The injury itself develops in several phases. During the earliest phase (i.e. minutes) of reperfusion, the increased oxidative stress and development of cardiomyocyte contracture seem to be the primary cause for necrotic cardiomyocyte injury (Piper *et al.*, 2003). Thereafter (i.e., minutes to hours), various additional causes can lead to a further increment of cell death either by necrosis or apoptosis, and vascular failure may further aggravate cardiomyocyte injury (Piper *et al.*, 2003).

Our first goal was to assess the impact of apoptotic and necrotic cell death in myocardial damage caused by cold, global I-R and the targets of Epo cardioprotective action in the transplanted heart. The obtained results indicate that, even when occurring, apoptosis plays a minor role in the I-R injury development in our model. In fact, the role and significance of apoptosis in the clinical situations is not well established (Murphy *et al.*, 2008; Tissier *et al.*, 2008). As mentioned already, cell death following ischemia-reperfusion has been reported to have features of both, apoptosis and necrosis. The precise proportion of each form of cell death may depend on the experimental model (adult vs. neonatal, cultured cells vs. *in vivo*) (Murphy *et al.*, 2008).

On one hand apoptosis is a programmed and well regulated process, resulting in formation of apoptotic bodies containing cellular components (Takemura *et al.*, 2006). The apoptotic bodies are cleared by phagocytosis, causing little or no inflammation response. It is well recognized that Epo can

interfere with the apoptotic process, and prevent cell death in different cell types, including cardiomyocytes (Parsa *et al.*, 2003; Ghezzi *et al.*, 2004; Burger *et al.*, 2006). However, we found that apoptosis does not play a major role in acute I-R in the experimental model used in this study. The apoptotic markers we have measured revealed little or no change within the period of observation (24h) and rhEpo administration failed to affect any of them (Paper 4).

On the other hand, necrotic cell death is characterized by cell swelling leading to irreversible rupture of the plasma membrane with release of cytosolic components and pronounced inflammatory response (Zong *et al.*, 2006; Golstein *et al.*, 2007). These are the features we have observed microscopically and when analyzing the ion/water balance in the grafts of the control group of recipients. We found that heterotopic heart transplantation resulted in tissue edema, depletion of cellular and plasma GSH levels, and an increase in the plasma levels of cardiac troponin T, suggesting cell membrane disruption and release of cytosolic components into the circulation (Paper 4). These observations imply that I-R injury observed in this model is accompanied by pronounced tissue necrosis.

Until recently, necrosis was considered to be an unregulated and irreversible process, with limited therapeutic significance. However, several studies suggested that necrosis can be regulated and that interventions can reduce necrotic cell death (Zong *et al.*, 2006; Golstein *et al.*, 2007). Our work revealed that Epo is a potent anti-necrotic factor, capable to reduce tissue damage right after the onset of reperfusion. Cytokine treatment resulted in a decrease of mechanical stress and the release of intracellular components in the blood stream already 5 min after it came in contact with the grafted heart (Paper 4). Epo administration also completely abolished reperfusion-induced oxidative stress and helped to restore the cellular redox potential, which was shifted during the ischemic phase to more oxidized (unpublished data, Paper 4).

In the heart, reactive oxygen species (ROS) are formed by various mechanisms, such as mitochondrial respiration, activated neutrophils, and xanthine oxidase. The activity of these oxidant-generating systems can be greatly enhanced in postischemic hearts and can overwhelm cellular defenses and induce tissue damage (Ambrosio *et al.*, 1999; Murphy *et al.*, 2008). For instance, ischemia alters the redox state of mitochondrial electron transport chain and promotes accumulation of xanthine (through adenosine triphosphate hydrolysis). Acute

deoxygenation also reduces NO production in the heart (Iwase *et al.*, 2007; Bogdanova *et al.*, 2008). These events lead to an increase in ROS production during the ischemic phase which is then enhanced manifold during reperfusion, when mitochondrial respiration resumes and the substrate supply to NOS, NADPH oxidase and xanthine oxidase gets restored (Ambrosio *et al.*, 1999; Murphy *et al.*, 2008). Furthermore, following reperfusion neutrophils accumulate within the previously ischemic area. Activated neutrophils release oxygen radicals in large amounts, with a maximum observed 6 h after the restoration of reperfusion in our experimental model (Ambrosio *et al.*, 1999; de Groot *et al.*, 2007). I-R accompanying heterotopic heart transplantation resulted in a pronounced and prolonged (until the end of the observation period) decrease in tissue-, erythrocyte- and plasma glutathione levels (Paper 3 and 4). GSH depletion in the blood of recipient animals and grafted hearts is a hallmark for local and systemic oxidative stress. rhEpo was able to restore GSH levels in the grafts and increase the basal glutathione content in non-transplanted (native) hearts. In addition, cytokine treatment resulted in a mitigation of the systemic oxidative stress, by restoring glutathione levels in the circulation (Paper 3 and 4). This effect of Epo has been described by us also in the isolated Epo-treated mouse erythrocytes (see section 5.4.5).

Taken together, our data suggest that in the settings of this *in vivo* model of global I-R injury, Epo plays a role mainly as acute, anti-necrotic, cytoprotective factor. rhEpo is able to decrease tissue damage and stress markers, along with general restoration of basal glutathione levels, a main anti-oxidant defense system in the organism.

6.4.4 Mechanism of Epo-induced cardioprotection – downstream targets and signaling

Epo-induced responses in endothelial cells and cardiomyocytes have been extensively described. They include activation of the STAT3, PI3K/Akt and MAPK signaling pathways and activation of eNOS (reviewed in (Noguchi *et al.*, 2008; Riksen *et al.*, 2008)). In line with these reports we observed transient activation of the PI3K/Akt pathway and eNOS phosphorylation in the vascular endothelium in the grafts and native hearts of rats treated with rhEpo (Papers 3 and 4). Phosphorylation of both Akt and eNOS at Ser-1177 can be observed already 5

minutes after the onset of reperfusion. Simultaneously NO_2^- plasma levels in the cytokine-treated animals were increased up to 1 h after the transplantation in comparison to control ones, indicating elevation in the NO production. Transient activation of PI3K/Akt pathway and upregulation in NO production was observed also in NRCs treated *in vitro* with rhEpo (Paper 3).

The three NO synthases (endothelial, inducible and neuronal) are largely distributed in the mammalian myocardium (including cardiomyocytes themselves), suggesting an important role of NO in heart function (Massion *et al.*, 2005). In general, NO has a number of physiologic effects, that could ameliorate I-R injury. The enhancement of NO production by L-Arg supplementation leads to an increase in post-ischemic blood flow (Szabo *et al.*, 1998), a suppression of inflammatory responses by decreasing leukocyte adhesion and expression of cell adhesion molecules (Adams *et al.*, 1997), and a quenching free radicals, particularly $*O_2^-$ (Takano *et al.*, 1998). The role of glycolysis is crucial at the start of reflow to restore ion-pumping functions in the sarcolemma and the sarcoplasmic reticulum (Jeremy *et al.*, 1993). L-arginine treated hearts show higher lactate production combined with lower phosphomonoester levels during reflow, which reflects a higher glycolytic activity that is protective by promoting a faster restoration of membrane function (Desrois *et al.*, 2000). It was shown that eNOS activation triggers PKC-mediated expression of inducible NOS (iNOS), resulting in opening of sarcolemmal K_{ATP} channels (Nandagopal *et al.*, 2001). iNOS and K_{ATP} activation were found to be involved in the early pharmacological preconditioning by Epo (Joyeux-Faure *et al.*, 2006). In addition, the NO-induced increase in cGMP within the myocytes would improve post-ischemic myocardial recovery by limiting Ca^{2+} overload (Mizuno *et al.*, 1998; Bredt, 2003). Vascular NO production has been shown to protect cardiomyocytes from apoptosis (Razavi *et al.*, 2005). The anti-apoptotic effects of NO are mediated by reducing oxidative stress via NADPH oxidase inhibition, by modulating the expression of protective genes such as heat shock protein 70 or Bcl-2, and by inhibiting caspase-3/caspase-8 activation through S-nitrosylation (Nandagopal *et al.*, 2001; Razavi *et al.*, 2005).

We have demonstrated that when applied iv, Epo only activates the endothelial eNOS, having no effect on the enzyme expressed in the myocytes (Papers 3 and 4). Moreover, the timing of Epo-induced effects on eNOS function (early activation followed by suppression of the vascular NO production in the late

time points) is essential for its cytoprotective role. At late time points (6 and 24 h after the onset of reperfusion) a strong eNOS phosphorylation in the blood vessel wall and very high NO_2^- plasma levels were detected in the control animals (Paper 3). The observed excessive activation of NO production is most probably due to the systemic inflammatory response (Stoclet *et al.*, 1998). The excessive production of NO in blood vessels is involved in circulatory failure that takes place in systemic inflammatory reactions and it may be cytotoxic for surrounding tissues. For these reasons, inhibition of NO overproduction has been proposed in the treatment of septic shock (Stoclet *et al.*, 1998). Intriguingly, Epo-treated animals showed decreased eNOS phosphorylation at Ser-1177 and lower NO_2^- plasma levels 6 and 24 h after the transplantation in comparison to controls. This dual effect of Epo on eNOS activity might be of particular importance for its cytoprotective properties (Paper 3). On one hand, the early activation of NO production might be beneficial for improvement of tissue perfusion and attenuation of the oxidative stress caused by I-R. On the other hand, the latter reduction in eNOS activity in Epo-treated animals could reduce the nitrosative stress accompanying the systemic inflammation.

Our data obtained form the experiments with mouse erythrocytes suggest that the Epo-induced NO production mediates the cytokine-triggered regulation of RBCs ion transport and cellular volume (see section 5.3.6). Further investigations using neonatal rat cardiomyocytes revealed that the activation of the NO production triggered by rhEpo treatment, modulates Na^+/K^+-ATPase activity (Papers 3 and 4). Similar coupling between NOS-mediated NO production and activity of the Na^+/K^+-ATPase in cerebellar granule cells was also documented (Petrushanko *et al.*, 2007). In agreement with these data, our *in vivo* experiments suggest that Epo was able to support the restoration of the Na^+ and water balance in the hearts, which was altered during ischemia and the early reperfusion phase. Since iv administered rhEpo binds only to the endothelial cells and does not pass through the vessel wall, endothelium-derived NO is most likely the responsible mediator for activation of Na^+/K^+-pump in cardiomyocytes. In addition, eNOS activation in the endothelium can affect tight junction-mediated endothelial resistance (Rao, 2008) and facilitate water drain from the interstitium to the circulation thus reducing hypoxic oedema. Therefore, an Epo-induced NO

production within the vascular endothelium could speed-up the restoration of normal ion/water balance in the cells and reduce I-R triggered tissue oedema.

In the cardiomyocytes, the normal Na^+/K^+-ATPase function is important for maintaining of the intracellular Ca^{2+} levels, since the principal mechanism of Ca^{2+} extrusion out of the cells (via Na^+/Ca^{2+}-exchanger) depends on the Na^+ gradient across the sarcolemma. One of the main cellular alterations during the ischemia period, include Ca^{2+} overload of cardiac cells caused by dissipated Na^+/K^+ gradients (Piper *et al.*, 2003; Buja, 2005; de Groot *et al.*, 2007). Restoration of oxidative phosphorylation upon reoxygenation is usually overrunning the restoration of ion homeostasis. Therefore, during the initial phase of reoxygenation, the cytosolic Ca^{2+} is still largely elevated and myofibrillar activation leads to uncontrolled, excessive force generation. This accelerated force generation causes hypercontraction injury characterized by severe cytoskeletal damage and cellular membrane rupture (Piper *et al.*, 2003; Murphy *et al.*, 2008). Facilitated restoration of the transmembrane Na^+ gradients in Epo-treated hearts would prevent excessive Ca^{2+} overload thereby reducing hypercontraction. The faster recovery of normal contractile activity would also reduce the mechanical stress that follows the restoration of perfusion. In accordance with this notion, we found that the Epo treatment resulted in a decrease of plasma levels of ANP and BNP, hallmarks of the ventricular mechanical overload.

Nitric oxide is not the only paracrine factor secreted by the endothelium upon Epo simulation. It was found previously that Epo treatment in renal patients is associated with changes in the levels of several paracrine regulators of cardiac function, such as endothelin-1 (ET-1) (Carlini *et al.*, 1993; Wada *et al.*, 1999) (Piuhola *et al.*, 2008), prostaglandins (Bode-Boger *et al.*, 1996), catecholamines (Ksiazek *et al.*, 2001) and agents of the renin–angiotensin system (Eggena *et al.*, 1991). However, most of these factors were reported to be released after prolonged a Epo application. As a negative feedback loop for the NO-induced effects one may suggest the activation of the cyclooxygenase responsible for the synthesis of vasoconstrictor prostanoids (ET-1 and prostaglandins). The latter can also produce superoxide anions (Katusic *et al.*, 1989) which deactivates endothelium-derived NO (Pacher *et al.*, 2007). The balance between the two opposite processes, eNOS and cyclooxygenase activation, depends strongly on the experimental conditions and Epo dosage, and defines the outcome of Epo

application (Jie *et al.*, 2006). Furthermore, in different pathological conditions L-arginine homeostasis could be altered (Wu *et al.*, 1999; Maarsingh *et al.*, 2004; Brunini *et al.*, 2005; Brunini *et al.*, 2006; Yang *et al.*, 2006) leading to impairment of the NO production (see section 5.4.5). Therefore a complete characterization of the time and dose dependence of Epo-induced responses in the vascular endothelial cells in physiological and pathophysiological conditions is required.

6.5 Conclusions and outlook II

The results of our study demonstrate that intravenous administration of rhEpo after the onset of ischemia, but prior to reperfusion protects the heart against cold global ischemia-reperfusion injury. The data we obtained, revealed that Epo is a potent anti-necrotic factor reducing tissue oedema, mechanical and oxidative stress triggered by I-R. We showed for the first time that iv administered rhEpo does not pass through the blood vessel wall and does not interact directly with the cardiomyocytes. The cytokine binds predominantly to the vascular endothelial cells, and its cardioprotective properties are most probably mediated by endothelium-derived paracrine factors. We demonstrated that after binding to the coronary endothelium, rhEpo activates the PI3K/Akt pathway and upregulates eNOS function via phosphorylation at Ser-1177. Our data imply that cardioprotective properties of rhEpo are at least partially mediated by nitric oxide released by the coronary endothelium. Further work is needed for the complete characterization of Epo-induced responses in the vascular endothelium and identification of the putative paracrine factors released by the coronary vessels. These data would help to optimize the protocols for clinical Epo application in order to achieve maximal cardioprotective efficiency of the cytokine and to avoid possible side effects of prolonged or intensive Epo-treatment.

Our *in vitro* experiments proved that neonatal cardiomyocytes can bind and respond to Epo treatment. Cytokine administration results in activation of the PI3K/Akt pathway, elevation of NO production and regulation of ion transport across the sarcolemma. It is known that Epo is required for the normal heart development. However, the role of Epo-induced changes in NO production and ion transport on the proliferation and differentiation of the embryonic myoblasts remain to be clarified.

The present findings cannot confirm or disapprove the ability of adult cardiomyocytes to bind Epo. A comprehensive study is needed to reveal the putative Epo-responses in adult cardiomyocytes. We don’t exclude the possibility that in some pathological conditions the blood vessel permeability might be altered (Plante, 2002; Weis, 2008) or endogenous, paracrine expression of Epo is induced (Jelkmann *et al.*, 2008). Therefore, it is important to evaluate the Epo-permeability of coronary vessels in different pathological and therapeutic conditions, and to clarify the physiological significance of endogenous Epo production in the heart.

7. REFERENCES

Abbrecht, P. H. and J. K. Littell (1972). "Plasma erythropoietin in men and mice during acclimatization to different altitudes." J Appl Physiol **32**(1): 54-8.

Adams, M. R., W. Jessup, et al. (1997). "L-arginine reduces human monocyte adhesion to vascular endothelium and endothelial expression of cell adhesion molecules." Circulation **95**(3): 662-8.

Alfrey, C. P., L. Rice, et al. (1997). "Neocytolysis: physiological down-regulator of red-cell mass." Lancet **349**(9062): 1389-90.

Ambrosio, G. and I. Tritto (1999). "Reperfusion injury: experimental evidence and clinical implications." Am Heart J **138**(2 Pt 2): S69-75.

Arcasoy, M. (2008). "The non-haematopoietic biological effects of erythropoietin." British Journal of Haematology **141**(1): 14-31.

Assandri, R., M. Egger, et al. (1999). "Erythropoietin modulates intracellular calcium in a human neuroblastoma cell line." J Physiol **516 (Pt 2)**: 343-52.

Baciu, I. and L. Ivanof (1983). "Erythropoietin interaction with the mature red cell membrane." Ann N Y Acad Sci **414**: 66-72.

Baciu, I., L. Ivanof, et al. (1985). "Erythropoietin binding to the red cell membranes." Physiologie **22**(4): 227-31.

Banerjee, D., M. Rodriguez, et al. (2000). "Exposure of endothelial cells to recombinant human erythropoietin induces nitric oxide synthase activity." Kidney Int **57**(5): 1895-904.

Barvitenko, N. N., N. C. Adragna, et al. (2005). "Erythrocyte signal transduction pathways, their oxygenation dependence and functional significance." Cell Physiol Biochem **15**(1-4): 1-18.

Baskurt, O. K. (1999). "The role of spleen in suppressing the rheological alterations in circulating blood." Clin Hemorheol Microcirc **20**(3): 181-8.

Baskurt, O. K. and H. J. Meiselman (2003). "Blood Rheology and Hemodynamics." Seminars in thrombosis and hemostasis. **29**(5): 435-50.

Behrooz, A. and F. Ismail-Beigi (1999). "Stimulation of Glucose Transport by Hypoxia: Signals and Mechanisms." News Physiol Sci **14**(3): 105-110.

Beleslin-Cokic, B. B., V. P. Cokic, et al. (2004). "Erythropoietin and hypoxia stimulate erythropoietin receptor and nitric oxide production by endothelial cells." Blood **104**(7): 2073-2080.

Beleslin-Cokic, B. B., V. P. Cokic, et al. (2004). "Erythropoietin and hypoxia stimulate erythropoietin receptor and nitric oxide production by endothelial cells." Blood **104**(7): 2073-80.

Bers, D. M. (2008). "Calcium cycling and signaling in cardiac myocytes." Annu Rev Physiol **70**: 23-49.

Birgegard, G. and B. Sandhagen (2001). "Erythropoetin treatment can increase 2,3-diphosphoglycerate levels in red blood cells." Scand J Clin Lab Invest **61**(5): 337-40.

Blanc, L., A. De Gassart, et al. (2005). "Exosome release by reticulocytes--an integral part of the red blood cell differentiation system." Blood Cells Mol Dis **35**(1): 21-6.

Bode-Boger, S. M., R. H. Boger, et al. (1996). "Recombinant human erythropoietin enhances vasoconstrictor tone via endothelin-1 and constrictor prostanoids." Kidney Int **50**(4): 1255-61.

Bogdanova, A., D. Mihov, et al. (2007). "Enhanced erythro-phagocytosis in polycythemic mice overexpressing erythropoietin." Blood **110**(2): 762-9.

Bogdanova, A., I. Petrushanko, et al. (2006). "Oxygen- and Redox-Induced Regulation of the Na/K ATPase." Current Enzyme Inhibition **2**: 37-59.

Bogdanova, A., J. Vogel, et al. (2008). Hypoxic Heart: Be a Trout, not a Rat. 4th CPB Meeting in Africa: MARA 2008, Maasai Mara Game Reserve Kenya, Medimond International Proceedings.

Bogoyevitch, M. A. (2004). "An update on the cardiac effects of erythropoietin cardioprotection by erythropoietin and the lessons learnt from studies in neuroprotection." Cardiovasc Res **63**(2): 208-216.

Boissel, J. P., W. R. Lee, et al. (1993). "Erythropoietin structure-function relationships. Mutant proteins that test a model of tertiary structure." J Biol Chem **268**(21): 15983-93.

Bolanos, J. P. C., Paula Garcıa-Nogales, Marıa Delgado-Esteban, Emilio Fernandez, Angeles Almeida (2004). "Regulation of glucose metabolism by nitrosative stress in neural cells." Molecular Aspects of Medicine **25**(1-2): 61-73.

Bor-Kucukatay, M., H. J. Meiselman, et al. (2005). "Modulation of density-fractionated RBC deformability by nitric oxide." Clinical Hemorheology and Microcirculation **33**(4): 363 - 367.

Bor-Kucukatay, M., R. B. Wenby, et al. (2003). "Effects of nitric oxide on red blood cell deformability." Am J Physiol Heart Circ Physiol **284**(5): H1577-1584.

Bredt, D. S. (2003). "Nitric oxide signaling specificity -- the heart of the problem 10.1242/jcs.00183." J Cell Sci **116**(1): 9-15.

Brewer, G. J. (1974). "2,3-DPG and erythrocyte oxygen affinity." Annu Rev Med **25**: 29-38.

Brines, M., G. Grasso, et al. (2004). "Erythropoietin mediates tissue protection through an erythropoietin and common {beta}-subunit heteroreceptor." Proceedings of the National Academy of Sciences **101**(41): 14907-14912.

Broudy, V. C., N. Lin, et al. (1991). "Erythropoietin receptor characteristics on primary human erythroid cells." Blood **77**(12): 2583-90.

Brunini, T., A. Resende, et al. (2005). "L-Arginine Availability as a Pathological Mechanism in Essential Hypertension, Chronic Renal and Heart Failure." Vascular Disease Prevention **2**: 37-51.

Brunini, T. M., N. B. Roberts, et al. (2006). "Activation of L-arginine transport in undialysed chronic renal failure and continuous ambulatory peritoneal dialysis patients." Clin Exp Pharmacol Physiol **33**(1-2): 114-8.

Buja, L. M. (2005). "Myocardial ischemia and reperfusion injury." Cardiovasc Pathol **14**(4): 170-5.

Bullard, A. J., P. Govewalla, et al. (2005). "Erythropoietin protects the myocardium against reperfusion injury in vitro and in vivo." Basic Res Cardiol **100**(5): 397-403.

Burger, D., M. Lei, et al. (2006). "Erythropoietin protects cardiomyocytes from apoptosis via up-regulation of endothelial nitric oxide synthase." Cardiovasc Res **72**(1): 51-9.

Cai, Z., D. J. Manalo, et al. (2003). "Hearts From Rodents Exposed to Intermittent Hypoxia or Erythropoietin Are Protected Against Ischemia-Reperfusion Injury." Circulation **108**(1): 79-85.

Cai, Z. and G. L. Semenza (2004). "Phosphatidylinositol-3-Kinase Signaling Is Required for Erythropoietin-Mediated Acute Protection Against Myocardial Ischemia/Reperfusion Injury." Circulation **109**(17): 2050-2053.

Calabrese, V., C. Mancuso, et al. (2007). "Nitric oxide in the central nervous system: neuroprotection versus neurotoxicity." Nat Rev Neurosci **8**(10): 766-75.

Calvillo, L., R. Latini, et al. (2003). "Recombinant human erythropoietin protects the myocardium from ischemia-reperfusion injury and promotes beneficial remodeling." Proc Natl Acad Sci U S A **100**(8): 4802-6.

Cardone, M. H., N. Roy, et al. (1998). "Regulation of cell death protease caspase-9 by phosphorylation." Science **282**(5392): 1318-21.

Carlini, R. G., A. S. Dusso, et al. (1993). "Recombinant human erythropoietin (rHuEPO) increases endothelin-1 release by endothelial cells." Kidney Int **43**(5): 1010-4.

Chakraborty, M., J. Ghosal, et al. (1986). "Effect of erythropoietin on the different ATPases and acetylcholinesterase of rat RBC membrane." Biochem Med Metab Biol **36**(2): 231-8.

Chakraborty, M., J. Ghosal, et al. (1988). "Effect of erythropoietin on membrane lipid peroxidation, superoxide dismutase, catalase, and glutathione peroxidase of rat RBC." Biochem Med Metab Biol **40**(1): 8-18.

Channon, K. M. and T. J. Guzik (2002). "Mechanisms of superoxide production in human blood vessels: relationship to endothelial dysfunction, clinical and genetic risk factors." J Physiol Pharmacol **53**(4 Pt 1): 515-24.

Chattopadhyay, A., T. D. Choudhury, et al. (2000). "Protective effect of erythropoietin on the oxidative damage of erythrocyte membrane by hydroxyl radical." Biochem Pharmacol **59**(4): 419-25.

Chen, C. and A. J. Sytkowski (2004). "Erythropoietin regulation of Raf-1 and MEK: evidence for a Ras-independent mechanism." Blood **104**(1): 73-80.

Cheung, J. Y. and B. A. Miller (2001). "Molecular mechanisms of erythropoietin signaling." Nephron **87**(3): 215-22.

Cohen, C. M. and P. Gascard (1992). "Regulation and post-translational modification of erythrocyte membrane and membrane-skeletal proteins." Semin Hematol **29**(4): 244-92.

Cossins, A. R. and J. S. Gibson (1997). "Volume-sensitive transport systems and volume homeostasis in vertebrate red blood cells." J Exp Biol **200**(2): 343-352.

Cristol, J. P., J. Y. Bosc, et al. (1997). "Erythropoietin and oxidative stress in haemodialysis: beneficial effects of vitamin E supplementation." Nephrol Dial Transplant **12**(11): 2312-7.

Cross, D. A., D. R. Alessi, et al. (1995). "Inhibition of glycogen synthase kinase-3 by insulin mediated by protein kinase B." Nature **378**(6559): 785-9.

d'Uscio, L. V., L. A. Smith, et al. (2007). "Essential Role of Endothelial Nitric Oxide Synthase in Vascular Effects of Erythropoietin." Hypertension **49**(5): 1142-1148.

De Franceschi, L., L. Ronzoni, et al. (2007). "K-CL co-transport plays an important role in normal and {beta} thalassemic erythropoiesis." Haematologica **92**(10): 1319-1326.

de Groot, H. and U. Rauen (2007). "Ischemia-reperfusion injury: processes in pathogenetic networks: a review." Transplant Proc **39**(2): 481-4.

de Jong, K. and F. A. Kuypers (2006). "Sulphydryl modifications alter scramblase activity in murine sickle cell disease." Br J Haematol **133**(4): 427-32.

de Oliveira Elias, M., W. Tavares de Lima, et al. (1999). "Nitric oxide modulates Na+, K+-ATPase activity through cyclic GMP pathway in proximal rat trachea." Eur J Pharmacol **367**(2-3): 307-14.

Dejam, A., C. J. Hunter, et al. (2005). "Erythrocytes are the major intravascular storage sites of nitrite in human blood." Blood **106**(2): 734-739.

Delpire, E. and S. R. Gullans (1994). "Cell volume and K+ transport during differentiation of mouse erythroleukemia cells." Am J Physiol **266**(2 Pt 1): C515-23.

Depping, R., K. Kawakami, et al. (2005). "Expression of the erythropoietin receptor in human heart." J Thorac Cardiovasc Surg **130**(3): 877-8.

Desrois, M., M. Sciaky, et al. (2000). "L-arginine during long-term ischemia: effects on cardiac function, energetic metabolism and endothelial damage." J Heart Lung Transplant **19**(4): 367-76.
Doyle, M. P. and J. W. Hoekstra (1981). "Oxidation of nitrogen oxides by bound dioxygen in hemoproteins." J Inorg Biochem **14**(4): 351-8.
Eggena, P., P. Willsey, et al. (1991). "Influence of recombinant human erythropoietin on blood pressure and tissue renin-angiotensin systems." Am J Physiol **261**(5 Pt 1): E642-6.
Eich, R. F., T. Li, et al. (1996). "Mechanism of NO-induced oxidation of myoglobin and hemoglobin." Biochemistry **35**(22): 6976-83.
Elliott, S., L. Busse, et al. (2006). "Anti-Epo receptor antibodies do not predict Epo receptor expression." Blood **107**(5): 1892-1895.
Fandrey, J. (2004). "Oxygen-dependent and tissue-specific regulation of erythropoietin gene expression." Am J Physiol Regul Integr Comp Physiol **286**(6): R977-88.
Fisher, J. W. (1997). "Erythropoietin: physiologic and pharmacologic aspects." Proc Soc Exp Biol Med **216**(3): 358-69.
Fisher, J. W. (2003). "Erythropoietin: physiology and pharmacology update." Exp Biol Med (Maywood) **228**(1): 1-14.
Flatman, P. W. (2002). "Regulation of Na-K-2Cl cotransport by phosphorylation and protein-protein interactions." Biochim Biophys Acta **1566**(1-2): 140-51.
Fleming, I. and R. Busse (2003). "Molecular mechanisms involved in the regulation of the endothelial nitric oxide synthase." Am J Physiol Regul Integr Comp Physiol **284**(1): R1-12.
Fliser, D., F. H. Bahlmann, et al. (2006). "Mechanisms of disease: erythropoietin--an old hormone with a new mission?" Nat Clin Pract Cardiovasc Med **3**(10): 563-72.
Foley, R. N. (2008). "Erythropoietin: physiology and molecular mechanisms." Heart Fail Rev **13**(4): 405-14.
Franke, T. F., C. P. Hornik, et al. (2003). "PI3K/Akt and apoptosis: size matters." Oncogene **22**(56): 8983-98.
Fraser, J. K., F. K. Lin, et al. (1988). "Expression of high affinity receptors for erythropoietin on human bone marrow cells and on the human erythroleukemic cell line, HEL." Exp Hematol **16**(10): 836-42.
Frein, D., S. Schildknecht, et al. (2005). "Redox regulation: a new challenge for pharmacology." Biochem Pharmacol **70**(6): 811-23.
Galinanes, M. and D. J. Hearse (1991). "Metabolic, functional, and histologic characterization of the heterotopically transplanted rat heart when used as a model for the study of long-term recovery from global ischemia." J Heart Lung Transplant **10**(1 Pt 1): 79-91.
Gallicchio, V. S. and M. J. Murphy, Jr. (1979). "Erythropoiesis in vitro. III. The role of potassium ions in erythroid colony formation." Exp Hematol **7**(5): 225-30.
Garcia-Nogales, P., A. Almeida, et al. (2003). "Peroxynitrite Protects Neurons against Nitric Oxide-mediated Apoptosis. A KEY ROLE FOR GLUCOSE-6-PHOSPHATE DEHYDROGENASE ACTIVITY IN NEUROPROTECTION." J. Biol. Chem. **278**(2): 864-874.
Ghezzi, P. and M. Brines (2004). "Erythropoietin as an antiapoptotic, tissue-protective cytokine." Cell Death Differ **11 Suppl 1**: S37-44.
Ghosal, J., M. Chakraborty, et al. (1987). "Effect of erythropoietin on the glucose transport of rat erythrocytes and bone marrow cells." Biochem Med Metab Biol **38**(2): 134-41.

Gladwin, M. T., J. H. Crawford, et al. (2004). "The biochemistry of nitric oxide, nitrite, and hemoglobin: role in blood flow regulation." Free Radical Biology and Medicine **36**(6): 707-717.

Gladwin, M. T. and D. B. Kim-Shapiro (2008). "The functional nitrite reductase activity of the heme-globins." Blood **112**(7): 2636-47.

Golstein, P. and G. Kroemer (2007). "Cell death by necrosis: towards a molecular definition." Trends Biochem Sci **32**(1): 37-43.

Gould, G. W., T. J. Jess, et al. (1994). "Evidence for a role of phosphatidylinositol 3-kinase in the regulation of glucose transport in Xenopus oocytes." J. Biol. Chem. **269**(43): 26622-26625.

Gross, A. W. and H. F. Lodish (2006). "Cellular trafficking and degradation of erythropoietin and novel erythropoiesis stimulating protein (NESP)." J Biol Chem **281**(4): 2024-32.

Hanada, M., J. Feng, et al. (2004). "Structure, regulation and function of PKB/AKT--a major therapeutic target." Biochim Biophys Acta **1697**(1-2): 3-16.

Hanlon, P. R., P. Fu, et al. (2005). "Mechanisms of erythropoietin-mediated cardioprotection during ischemia-reperfusion injury: role of protein kinase C and phosphatidylinositol 3-kinase signaling." FASEB J.: 04-3545fje.

Hayes, J. D. and L. I. McLellan (1999). "Glutathione and glutathione-dependent enzymes represent a co-ordinately regulated defence against oxidative stress." Free Radic Res **31**(4): 273-300.

Heeschen, C., A. Aicher, et al. (2003). "Erythropoietin is a potent physiologic stimulus for endothelial progenitor cell mobilization." Blood **102**(4): 1340-6.

Hirata, A., T. Minamino, et al. (2005). "Erythropoietin just before reperfusion reduces both lethal arrhythmias and infarct size via the phosphatidylinositol-3 kinase-dependent pathway in canine hearts." Cardiovasc Drugs Ther **19**(1): 33-40.

Iwase, H., E. Robin, et al. (2007). "Nitric oxide during ischemia attenuates oxidant stress and cell death during ischemia and reperfusion in cardiomyocytes." Free Radic Biol Med **43**(4): 590-9.

Jacob, H. S., J. W. Eaton, et al. (1975). "Shortened red blood cell survival in uremic patients: beneficial and deleterious effects of dialysis." Kidney Int Suppl(2): 139-43.

Jacobs, K., C. Shoemaker, et al. (1985). "Isolation and characterization of genomic and cDNA clones of human erythropoietin." Nature **313**(6005): 806-10.

Jelkmann, W. (2004). "Molecular biology of erythropoietin." Intern Med **43**(8): 649-59.

Jelkmann, W. (2007). "Erythropoietin after a century of research: younger than ever." Eur J Haematol **78**(3): 183-205.

Jelkmann, W., J. Bohlius, et al. (2008). "The erythropoietin receptor in normal and cancer tissues." Crit Rev Oncol Hematol **67**(1): 39-61.

Jeremy, R. W., G. Ambrosio, et al. (1993). "The functional recovery of post-ischemic myocardium requires glycolysis during early reperfusion." J Mol Cell Cardiol **25**(3): 261-76.

Jie, K. E., M. C. Verhaar, et al. (2006). "Erythropoietin and the cardiorenal syndrome: cellular mechanisms on the cardiorenal connectors." Am J Physiol Renal Physiol **291**(5): F932-944.

Jindal, H. K., Z. Ai, et al. (1996). "Specific loss of protein kinase activities in senescent erythrocytes." Blood **88**(4): 1479-87.

Johnstone, R. M., M. Adam, et al. (1987). "Vesicle formation during reticulocyte maturation. Association of plasma membrane activities with released vesicles (exosomes)." J. Biol. Chem. **262**(19): 9412-9420.

Joyeux-Faure, M., A. Ramond, et al. (2006). "Early pharmacological preconditioning by erythropoietin mediated by inducible NOS and mitochondrial ATP-dependent potassium channels in the rat heart." Fundam Clin Pharmacol **20**(1): 51-6.

Kanagy, N. L., M. F. Perrine, et al. (2003). "Erythropoietin administration in vivo increases vascular nitric oxide synthase expression." J Cardiovasc Pharmacol **42**(4): 527-33.

Kashiwagi, A., T. P. Huecksteadt, et al. (1983). "The regulation of glucose transport by cAMP stimulators via three different mechanisms in rat and human adipocytes." J. Biol. Chem. **258**(22): 13685-13692.

Katusic, Z. S. and P. M. Vanhoutte (1989). "Superoxide anion is an endothelium-derived contracting factor." Am J Physiol **257**(1 Pt 2): H33-7.

Khandelwal, S. and R. K. Saxena (2008). "A role of phosphatidylserine externalization in clearance of erythrocytes exposed to stress but not in eliminating aging populations of erythrocyte in mice." Exp Gerontol **43**(8): 764-70.

Kim-Shapiro, D. B., A. N. Schechter, et al. (2006). "Unraveling the Reactions of Nitric Oxide, Nitrite, and Hemoglobin in Physiology and Therapeutics." Arterioscler Thromb Vasc Biol **26**(4): 697-705.

Klatt, P. and S. Lamas (2000). "Regulation of protein function by S-glutathiolation in response to oxidative and nitrosative stress." European Journal of Biochemistry **267**(16): 4928-4944.

Kleinbongard, P., A. Dejam, et al. (2003). "Plasma nitrite reflects constitutive nitric oxide synthase activity in mammals." Free Radic Biol Med. **35**(7): 790-796

Kleinbongard, P., R. Schulz, et al. (2006). "Red blood cells express a functional endothelial nitric oxide synthase." Blood **107**(7): 2943-2951.

Klingmuller, U., U. Lorenz, et al. (1995). "Specific recruitment of SH-PTP1 to the erythropoietin receptor causes inactivation of JAK2 and termination of proliferative signals." Cell **80**(5): 729-38.

Kosaka, H. and A. Seiyama (1996). "Physiological role of nitric oxide as an enhancer of oxygen transfer from erythrocytes to tissues." Biochem Biophys Res Commun **218**(3): 749-52.

Koury, M. J. and M. C. Bondurant (1992). "The molecular mechanism of erythropoietin action." Eur J Biochem **210**(3): 649-63.

Krzyzanski, W. and J. J. Perez-Ruixo (2007). "An assessment of recombinant human erythropoietin effect on reticulocyte production rate and lifespan distribution in healthy subjects." Pharm Res **24**(4): 758-72.

Ksiazek, A., W. T. Zaluska, et al. (2001). "Effect of recombinant human erythropoietin on adrenergic activity in normotensive hemodialysis patients." Clin Nephrol **56**(2): 104-10.

Lacombe, C. and P. Mayeux (1999). "The molecular biology of erythropoietin." Nephrol Dial Transplant **14 Suppl 2**: 22-8.

Lambert, I. H., E. K. Hoffmann, et al. (2008). "Cell volume regulation: physiology and pathophysiology." Acta Physiologica **194**(4): 255-282.

Li, F., Z. Z. Chong, et al. (2004). "Erythropoietin on a tightrope: balancing neuronal and vascular protection between intrinsic and extrinsic pathways." Neurosignals **13**(6): 265-89.

Lin, F. K., C. H. Lin, et al. (1986). "Monkey erythropoietin gene: cloning, expression and comparison with the human erythropoietin gene." Gene **44**(2-3): 201-9.

Lin, F. K., S. Suggs, et al. (1985). "Cloning and expression of the human erythropoietin gene." Proc Natl Acad Sci U S A **82**(22): 7580-4.

Linde, T., B. Sandhagen, et al. (1992). "Impaired erythrocyte fluidity during treatment of renal anaemia with erythropoietin." J Intern Med **231**(6): 601-6.
Lipsic, E., R. G. Schoemaker, et al. (2006). "Protective Effects of Erythropoietin in Cardiac Ischemia: From Bench to Bedside." Journal of the American College of Cardiology **48**(11): 2161-2167.
Lopez Ongil, S. L., M. Saura, et al. (1996). "Recombinant human erythropoietin does not regulate the expression of endothelin-1 and constitutive nitric oxide synthase in vascular endothelial cells." Exp Nephrol **4**(1): 37-42.
Loughrey, C. M., I. S. Young, et al. (1994). "Oxidative stress in haemodialysis." Qjm **87**(11): 679-83.
Lutz, H. U. (2004). "Innate immune and non-immune mediators of erythrocyte clearance." Cell Mol Biol (Noisy-le-grand) **50**(2): 107-16.
Lutz, H. U., F. Bussolino, et al. (1987). "Naturally occurring anti-band-3 antibodies and complement together mediate phagocytosis of oxidatively stressed human erythrocytes." Proc Natl Acad Sci U S A **84**(21): 7368-72.
Lutz, H. U., S. Fasler, et al. (1988). "Naturally occurring anti-band 3 antibodies and complement in phagocytosis of oxidatively-stressed and in clearance of senescent red cells." Blood Cells **14**(1): 175-203.
Lutz, H. U., S. C. Liu, et al. (1977). "Release of spectrin-free vesicles from human erythrocytes during ATP depletion. I. Characterization of spectrin-free vesicles." J Cell Biol **73**(3): 548-60.
Lutz, H. U., P. Stammler, et al. (1992). "Density separation of human red blood cells on self forming Percoll gradients: correlation with cell age." Biochim Biophys Acta **1116**(1): 1-10.
Maarsingh, H., J. de Boer, et al. (2004). "Heparin normalizes allergen-induced nitric oxide deficiency and airway hyperresponsiveness." Br J Pharmacol **142**(8): 1293-9.
Maiese, K., Z. Z. Chong, et al. (2008). "Erythropoietin: elucidating new cellular targets that broaden therapeutic strategies." Prog Neurobiol **85**(2): 194-213.
Maiese, K., F. Li, et al. (2005). "New avenues of exploration for erythropoietin." Jama **293**(1): 90-5.
Maletic, S. D., L. M. Dragicevic-Djokovic, et al. (2000). "Effects of nitric oxide donors on energy metabolism of rat erythrocytes." J Environ Pathol Toxicol Oncol **19**(4): 383-90.
Mallozzi, C., A. M. Di Stasi, et al. (1997). "Peroxynitrite modulates tyrosine-dependent signal transduction pathway of human erythrocyte band 3." FASEB J. **11**(14): 1281-1290.
Mao, W., C. Iwai, et al. (2008). "Darbepoetin alfa exerts a cardioprotective effect in autoimmune cardiomyopathy via reduction of ER stress and activation of the PI3K/Akt and STAT3 pathways." J Mol Cell Cardiol **45**(2): 250-60.
Maouche, L., C. Tournamille, et al. (1991). "Cloning of the gene encoding the human erythropoietin receptor." Blood **78**(10): 2557-63.
Mason, J. M., B. K. Beattie, et al. (2000). "The SH2 inositol 5-phosphatase Ship1 is recruited in an SH2-dependent manner to the erythropoietin receptor." J Biol Chem **275**(6): 4398-406.
Massion, P. B., M. Pelat, et al. (2005). "Regulation of the mammalian heart function by nitric oxide." Comp Biochem Physiol A Mol Integr Physiol **142**(2): 144-50.
Matsuura, T., Y. Kanayama, et al. (1993). "cAMP-induced changes of intracellular free Mg2+ levels in human erythrocytes." Biochim Biophys Acta **1220**(1): 31-6.
Mattson, M. P. and S. L. Chan (2003). "Calcium orchestrates apoptosis." Nat Cell Biol **5**(12): 1041-3.

McDonald, J. D., F. K. Lin, et al. (1986). "Cloning, sequencing, and evolutionary analysis of the mouse erythropoietin gene." Mol Cell Biol **6**(3): 842-8.
Miki, Y., T. Tazawa, et al. (2007). "Clearance of oxidized erythrocytes by macrophages: involvement of caspases in the generation of clearance signal at band 3 glycoprotein." Biochem Biophys Res Commun **363**(1): 57-62.
Minetti, G. and P. S. Low (1997). "Erythrocyte signal transduction pathways and their possible functions." Curr Opin Hematol **4**(2): 116-21.
Mizuno, T., M. Watanabe, et al. (1998). "L-arginine, a nitric oxide precursor, attenuates ischemia-reperfusion injury by inhibiting inositol-1,4,5-triphosphate." J Thorac Cardiovasc Surg **115**(4): 931-6.
Moncada, S., R. Palmer, et al. (1991). "Nitric oxide: physiology, pathophysiology, and pharmacology." Pharmacol Rev **43**(2): 109-142.
Moon, C., M. Krawczyk, et al. (2003). "Erythropoietin reduces myocardial infarction and left ventricular functional decline after coronary artery ligation in rats." Proc Natl Acad Sci U S A **100**(20): 11612-7.
Moon, C., M. Krawczyk, et al. (2005). "Cardioprotection by recombinant human erythropoietin following acute experimental myocardial infarction: dose response and therapeutic window." Cardiovasc Drugs Ther **19**(4): 243-50.
Mount, P., B. Kemp, et al. (2006). "Regulation of endothelial and myocardial NO synthesis by multi-site eNOS phosphorylation " J Mol Cell Cardiol. **42**(2): 271-279.
Muller, B., A. L. Kleschyov, et al. (2002). "Nitric Oxide Transport and Storage in the Cardiovascular System." Ann NY Acad Sci **962**(1): 131-139.
Munzel, T., A. Daiber, et al. (2005). "Vascular Consequences of Endothelial Nitric Oxide Synthase Uncoupling for the Activity and Expression of the Soluble Guanylyl Cyclase and the cGMP-Dependent Protein Kinase." Arterioscler Thromb Vasc Biol **25**(8): 1551-1557.
Murphy, E. and C. Steenbergen (2008). "Mechanisms Underlying Acute Protection From Cardiac Ischemia-Reperfusion Injury." Physiol. Rev. **88**(2): 581-609.
Myssina, S., S. M. Huber, et al. (2003). "Inhibition of erythrocyte cation channels by erythropoietin." J Am Soc Nephrol **14**(11): 2750-7.
Nandagopal, K., T. M. Dawson, et al. (2001). "Critical Role for Nitric Oxide Signaling in Cardiac and Neuronal Ischemic Preconditioning and Tolerance." J Pharmacol Exp Ther **297**(2): 474-478.
Nemeth, I., S. Turi, et al. (2000). "Vitamin E alleviates the oxidative stress of erythropoietin in uremic children on hemodialysis." Pediatr Nephrol **14**(1): 13-7.
Ng, T., G. Marx, et al. (2003). "Recombinant erythropoietin in clinical practice." Postgrad Med J **79**(933): 367-376.
Nguyen-Khoa, T., Z. A. Massy, et al. (2001). "Oxidative stress and haemodialysis: role of inflammation and duration of dialysis treatment." Nephrol. Dial. Transplant. **16**(2): 335-340.
Nicolay, J. P., G. Liebig, et al. (2008). "Inhibition of suicidal erythrocyte death by nitric oxide." Pflugers Arch **456**(2): 293-305.
Nishihara, M., T. Miura, et al. (2006). "Erythropoietin affords additional cardioprotection to preconditioned hearts by enhanced phosphorylation of glycogen synthase kinase-3beta." Am J Physiol Heart Circ Physiol **291**(2): H748-755.
Noguchi, C. T., L. Wang, et al. (2008). "Survival and proliferative roles of erythropoietin beyond the erythroid lineage." Expert Rev Mol Med **10**: e36.
Ogilvie, M., X. Yu, et al. (2000). "Erythropoietin stimulates proliferation and interferes with differentiation of myoblasts." J Biol Chem **275**(50): 39754-61.

Ono, K. and E. S. Lindsey (1969). "Improved technique of heart transplantation in rats." J Thorac Cardiovasc Surg **57**(2): 225-9.
Oonishi, T., K. Sakashita, et al. (1997). "Regulation of red blood cell filterability by Ca2+ influx and cAMP-mediated signaling pathways." Am J Physiol Cell Physiol **273**(6): C1828-1834.
Pacher, P., J. S. Beckman, et al. (2007). "Nitric Oxide and Peroxynitrite in Health and Disease." Physiol. Rev. %R 10.1152/physrev.00029.2006 **87**(1): 315-424.
Parsa, C. J., A. Matsumoto, et al. (2003). "A novel protective effect of erythropoietin in the infarcted heart." J Clin Invest **112**(7): 999-1007.
Pastore, A., G. Federici, et al. (2003). "Analysis of glutathione: implication in redox and detoxification." Clin Chim Acta **333**(1): 19-39.
Pawloski, J. R., D. T. Hess, et al. (2001). "Export by red blood cells of nitric oxide bioactivity." Nature **409**(6820): 622-6.
Pawloski, J. R. and J. S. Stamler (2002). "Nitric oxide in RBCs." Transfusion **42**(12): 1603-9.
Pellegrino, C. M., A. C. Rybicki, et al. (1998). "Molecular identification and expression of erythroid K:Cl cotransporter in human and mouse erythroleukemic cells." Blood Cells Mol Dis **24**(1): 31-40.
Peter Klatt, S. L. (2000). "Regulation of protein function by S-glutathiolation in response to oxidative and nitrosative stress." European Journal of Biochemistry **267**(16): 4928-4944.
Petrov, V. and P. Lijnen (1996). "Regulation of human erythrocyte Na+/H+ exchange by soluble and particulate guanylate cyclase." Am J Physiol Cell Physiol **271**(5): C1556-1564.
Petrushanko, I. Y., N. B. Bogdanov, et al. (2007). "Oxygen-induced Regulation of Na/K ATPase in Cerebellar Granule Cells." J. Gen. Physiol. **130**(4): 389-398.
Piper, H. M., K. Meuter, et al. (2003). "Cellular mechanisms of ischemia-reperfusion injury." Ann Thorac Surg **75**(2): S644-8.
Piuhola, J., R. Kerkela, et al. (2008). "Direct cardiac actions of erythropoietin (EPO): effects on cardiac contractility, BNP secretion and ischaemia/reperfusion injury." Clin Sci (Lond) **114**(4): 293-304.
Plante, G. E. (2002). "Vascular response to stress in health and disease." Metabolism **51**(6 Suppl 1): 25-30.
Powell, J. S., K. L. Berkner, et al. (1986). "Human erythropoietin gene: high level expression in stably transfected mammalian cells and chromosome localization." Proc Natl Acad Sci U S A **83**(17): 6465-9.
Rafiee, P., Y. Shi, et al. (2005). "Erythropoietin protects the infant heart against ischemia-reperfusion injury by triggering multiple signaling pathways." Basic Res Cardiol **100**(3): 187-97.
Ramzy, D., V. Rao, et al. (2006). "Elevated Endothelin-1 Levels Impair Nitric Oxide Homeostasis Through a PKC-Dependent Pathway." Circulation **114**(1_suppl): I-319-326.
Rao, R. (2008). "Oxidative stress-induced disruption of epithelial and endothelial tight junctions." Front Biosci **13**: 7210-26.
Ratajczak, J., M. Majka, et al. (2001). "Biological significance of MAPK, AKT and JAK-STAT protein activation by various erythropoietic factors in normal human early erythroid cells." Br J Haematol **115**(1): 195-204.
Razavi, H. M., J. A. Hamilton, et al. (2005). "Modulation of apoptosis by nitric oxide: implications in myocardial ischemia and heart failure." Pharmacol Ther **106**(2): 147-62.

Rice, L., W. Ruiz, et al. (2001). "Neocytolysis on descent from altitude: a newly recognized mechanism for the control of red cell mass." Ann Intern Med **134**(8): 652-6.

Riksen, N. P., D. J. Hausenloy, et al. (2008). "Erythropoietin: ready for prime-time cardioprotection." Trends Pharmacol Sci **29**(5): 258-67.

Risso, A., M. Turello, et al. (2007). "Red blood cell senescence and neocytolysis in humans after high altitude acclimatization." Blood Cells, Molecules, and Diseases **38**(2): 83-92.

Sahu, S. K., S. N. Gummadi, et al. (2007). "Phospholipid scramblases: an overview." Arch Biochem Biophys **462**(1): 103-14.

Santhanam, A. V., L. V. d'Uscio, et al. (2008). "Activation of endothelial nitric oxide synthase is critical for erythropoietin-induced mobilization of progenitor cells." Peptides **29**(8): 1451-5.

Santhanam, A. V. R. and Z. S. Katusic (2006). "Erythropoietin and cerebral vascular protection: role of nitric oxide." Acta Pharmacologica Sinica **27**(11): 1389-1394.

Sasaki, R. (2003). "Pleiotropic Functions of Erythropoietin." Internal Medicine **42**(2): 142-149.

Sasaki, R., S. Masuda, et al. (2000). "Erythropoietin: multiple physiological functions and regulation of biosynthesis." Biosci Biotechnol Biochem **64**(9): 1775-93.

Sawada, K., S. B. Krantz, et al. (1990). "Purification of human blood burst-forming units-erythroid and demonstration of the evolution of erythropoietin receptors." J Cell Physiol **142**(2): 219-30.

Schaefer, A., M. Magocsi, et al. (1997). "Signalling mechanisms in erythropoiesis: the enigmatic role of calcium." Cell Signal **9**(7): 483-95.

Schafer, B. W. and J. C. Perriard (1988). "Intracellular targeting of isoproteins in muscle cytoarchitecture." J Cell Biol **106**(4): 1161-70.

Schafer, F. Q. and G. R. Buettner (2001). "Redox environment of the cell as viewed through the redox state of the glutathione disulfide/glutathione couple." Free Radic Biol Med **30**(11): 1191-212.

Semenza, G. L. (2001). "HIF-1, O(2), and the 3 PHDs: how animal cells signal hypoxia to the nucleus." Cell **107**(1): 1-3.

Sharma, R., Y. Yang, et al. (2004). "Antioxidant Role of Glutathione S-Transferases: Protection Against Oxidant Toxicity and Regulation of Stress-Mediated Apoptosis." Antioxidants & Redox Signaling **6**(2): 289-300.

Shi, Y., P. Rafiee, et al. (2004). "Acute cardioprotective effects of erythropoietin in infant rabbits are mediated by activation of protein kinases and potassium channels." Basic Research in Cardiology **99**(3): 173-182.

Simons, T. J. (1988). "Calcium and neuronal function." Neurosurg Rev **11**(2): 119-29.

Snyder, L. M., G. Fairbanks, et al. (1985). "Properties and characterization of vesicles released by young and old human red cells." Br J Haematol **59**(3): 513-22.

Socolovsky, M., A. E. Fallon, et al. (1999). "Fetal anemia and apoptosis of red cell progenitors in Stat5a-/-5b-/- mice: a direct role for Stat5 in Bcl-X(L) induction." Cell **98**(2): 181-91.

Spivak, J. L., L. S. Avedissian, et al. (1996). "Isolation of the full-length murine erythropoietin receptor using a baculovirus expression system." Blood **87**(3): 926-937.

Sprague, R. S., A. H. Stephenson, et al. (2007). "Red not dead: signaling in and from erythrocytes." Trends Endocrinol Metab **18**(9): 350-5.

Starzyk, D., R. Korbut, et al. (1999). "Effects of nitric oxide and prostacyclin on deformability and aggregability of red blood cells of rats ex vivo and in vitro." J Physiol Pharmacol **50**(4): 629-37.
Stephanou, A. (2004). "Role of STAT-1 and STAT-3 in ischaemia/reperfusion injury." Journal of Cellular and Molecular Medicine **8**(4): 519-525.
Stepuro, T. L. and V. V. Zinchuk (2006). "Nitric oxide effect on the hemoglobin-oxygen affinity." J Physiol Pharmacol **57**(1): 29-38.
Stoclet, J. C., B. Muller, et al. (1998). "Overproduction of nitric oxide in pathophysiology of blood vessels." Biochemistry (Mosc) **63**(7): 826-32.
Stuckmann, I., S. Evans, et al. (2003). "Erythropoietin and retinoic acid, secreted from the epicardium, are required for cardiac myocyte proliferation." Dev Biol **255**(2): 334-49.
Sui, X., S. B. Krantz, et al. (1998). "Synergistic activation of MAP kinase (ERK1/2) by erythropoietin and stem cell factor is essential for expanded erythropoiesis." Blood **92**(4): 1142-9.
Suzuki, T. and G. L. Dale (1989). "Membrane proteins in senescent erythrocytes." Biochem J **257**(1): 37-41.
Szabo, G., S. Bahrle, et al. (1998). "L-arginine: effect on reperfusion injury after heart transplantation." World J Surg **22**(8): 791-7; discussion 797-8.
Takano, H., X.-L. Tang, et al. (1998). "Nitric Oxide Donors Induce Late Preconditioning Against Myocardial Stunning and Infarction in Conscious Rabbits via an Antioxidant-Sensitive Mechanism." Circ Res **83**(1): 73-84.
Takemura, G. and H. Fujiwara (2006). "Morphological aspects of apoptosis in heart diseases." Journal of cellular and molecular medicine **10**(1): 56-75.
Therien, A. G. and R. Blostein (2000). "Mechanisms of sodium pump regulation." Am J Physiol Cell Physiol **279**(3): C541-66.
Tilbrook, P. A. and S. P. Klinken (1999). "Erythropoietin and erythropoietin receptor." Growth Factors **17**(1): 25-35.
Tissier, R., A. Berdeaux, et al. (2008). "Making the heart resistant to infarction: how can we further decrease infarct size?" Front Biosci **13**: 284-301.
Tramontano, A. F., R. Muniyappa, et al. (2003). "Erythropoietin protects cardiac myocytes from hypoxia-induced apoptosis through an Akt-dependent pathway." Biochem Biophys Res Commun **308**(4): 990-4.
Trial, J. and L. Rice (2004). "Erythropoietin withdrawal leads to the destruction of young red cells at the endothelial-macrophage interface." Curr Pharm Des **10**(2): 183-90.
Tuvia, S., A. Moses, et al. (1999). "beta-Adrenergic agonists regulate cell membrane fluctuations of human erythrocytes." J Physiol **516**(3): 781-792.
Urao, N., M. Okigaki, et al. (2006). "Erythropoietin-mobilized endothelial progenitors enhance reendothelialization via Akt-endothelial nitric oxide synthase activation and prevent neointimal hyperplasia." Circ Res **98**(11): 1405-13.
van der Meer, P., E. Lipsic, et al. (2004). "Erythropoietin improves left ventricular function and coronary flow in an experimental model of ischemia-reperfusion injury." Eur J Heart Fail **6**(7): 853-9.
Vogel, J., I. Kiessling, et al. (2003). "Transgenic mice overexpressing erythropoietin adapt to excessive erythrocytosis by regulating blood viscosity." Blood **102**(6): 2278-84.
Wada, Y., H. Matsuoka, et al. (1999). "Erythropoietin impairs endothelium-dependent vasorelaxation through cyclooxygenase-dependent mechanisms in humans." Am J Hypertens **12**(10 Pt 1): 980-7.
Wald, M. R., E. S. Borda, et al. (1996). "Mitogenic effect of erythropoietin on neonatal rat cardiomyocytes: signal transduction pathways." J Cell Physiol **167**(3): 461-8.

Walrafen, P., F. Verdier, et al. (2005). "Both proteasomes and lysosomes degrade the activated erythropoietin receptor
10.1182/blood-2004-03-1216." Blood **105**(2): 600-608.
Wang, G. L. and G. L. Semenza (1993). "General involvement of hypoxia-inducible factor 1 in transcriptional response to hypoxia." Proc Natl Acad Sci U S A **90**(9): 4304-8.
Wang, X. Q. and N. D. Vaziri (1999). "Erythropoietin Depresses Nitric Oxide Synthase Expression by Human Endothelial Cells." Hypertension **33**(3): 894-899.
Waugh, R. E., M. Narla, et al. (1992). "Rheologic properties of senescent erythrocytes: loss of surface area and volume with red blood cell age." Blood **79**(5): 1351-8.
Weis, S. M. (2008). "Vascular permeability in cardiovascular disease and cancer." Curr Opin Hematol **15**(3): 243-9.
Wheatley, D. J. (2002). "The potential of the heterotopic rat heart transplant model." Transplantation **73**(9): 1382-3.
Wickrema, A., S. Krantz, et al. (1992). "Differentiation and erythropoietin receptor gene expression in human erythroid progenitor cells " Blood **80**(8): 1940-1949.
Wiley, J. S. and C. C. Shaller (1977). "Selective loss of calcium permeability on maturation of reticulocytes." J Clin Invest **59**(6): 1113-9.
Wojchowski, D. M., R. C. Gregory, et al. (1999). "Signal transduction in the erythropoietin receptor system." Exp Cell Res **253**(1): 143-56.
Woo, S., W. Krzyzanski, et al. (2007). "Target-mediated pharmacokinetic and pharmacodynamic model of recombinant human erythropoietin (rHuEPO)." J Pharmacokinet Pharmacodyn **34**(6): 849-68.
Wright, G. L., P. Hanlon, et al. (2004). "Erythropoietin receptor expression in adult rat cardiomyocytes is associated with an acute cardioprotective effect for recombinant erythropoietin during ischemia-reperfusion injury." FASEB J.: 03-1289fje.
Wu, G., N. E. Flynn, et al. (1999). "Dietary Protein or Arginine Deficiency Impairs Constitutive and Inducible Nitric Oxide Synthesis by Young Rats." J. Nutr. **129**(7): 1347-1354.
Wu, H., S. H. Lee, et al. (1999). "Inactivation of erythropoietin leads to defects in cardiac morphogenesis." Development **126**(16): 3597-3605.
Wu, X. C., E. J. Johns, et al. (1999). "Relationship between erythropoietin and nitric oxide in the contraction of rat renal arcuate arteries and human umbilical vein endothelial cells." Clin Sci (Lond) **97**(4): 413-9.
Yamamoto, M., K. Koshimura, et al. (2004). "Effect of erythropoietin on nitric oxide production in the rat hippocampus using in vivo brain microdialysis." Neuroscience **128**(1): 163-8.
Yang, Z. and D. M. Kaye (2006). "Endothelial dysfunction and impaired L-arginine transport in hypertension and genetically predisposed normotensive subjects." Trends Cardiovasc Med **16**(4): 118-24.
Yellon, D. M. and D. J. Hausenloy (2007). "Myocardial Reperfusion Injury." N Engl J Med **357**(11): 1121-1135.
Youssoufian, H., G. Longmore, et al. (1993). "Structure, function, and activation of the erythropoietin receptor." Blood **81**(9): 2223-36.
Zong, W. X. and C. B. Thompson (2006). "Necrotic death as a cell fate." Genes Dev **20**(1): 1-15.

8. PAPERS AND MANUSCRIPTS

8.1 Paper 1 (submitted manuscript)

Erythropoietin activates nitric oxide synthase in murine erythrocytes

Manuscript #	C-00543-2008
Current Revision #	0
Other Version	C-00543-2008R1
Submission Date	22nd Oct 08 09:22:52
Current Stage	Under revision
Title	Erythropoietin activates nitric oxide synthase in murine erythrocytes
Running Title	Epo activates eNOS in mouse erythrocytes
Manuscript Type	Research Article
Special Section	None
Category	Receptors and Signal Transduction
Corresponding Author	Dr. Anna Bogdanova (University of Zurich)
Contributing Authors	Mr. Deyan Mihov , Prof. Johannes Vogel , Prof. Max Gassmann

Associate Editor	Dr. Sophie Lotersztajn
Key Words	red blood cells, PI3K/Akt pathway, NO production, redox state, Epo
Decision	Major Revision / 27th Nov 08
Mandatory Submission Form	DM

Database Deposition	N/A
Copyright Release Date	23rd Oct 08

Erythropoietin activates nitric oxide synthase in murine erythrocytes

Authors: **Deyan Mihov, Johannes Vogel, Max Gassmann, Anna Bogdanova**

Institution: **Institute of Veterinary Physiology, Vetsuisse Faculty and Zürich Center for Integrative Human Physiology (ZIHP), University of Zürich, Switzerland**

Running title: Epo activates eNOS in mouse erythrocytes

Corresponding author:

Dr. Anna Yu. Bogdanova

Institute of Veterinary Physiology, Zurich University

Winterthurerstrasse 260

CH-8057, Zürich

Switzerland

Tel: +41 44 6358811

Fax: +41 44 6358932

Email: annab@access.uzh.ch

ABSTRACT

Erythropoietin (Epo) is the main regulator of erythrocyte production and a potent cytoprotective factor. It was suggested that some of Epo cytoprotective properties are due to its regulation of nitric oxide (NO) production. Recently, functionally active endothelial type NO synthase was discovered in mature murine and human red blood cells (RBC-eNOS). The goal of the present study was to characterize the effect of physiologic and therapeutic doses of Epo on RBC-eNOS function. We found that recombinant human Epo (rHuEpo) binds specifically to mouse erythrocytes. Epo binding sites are not equally distributed through the RBC population but prevail in reticulocytes and young erythrocytes with about 105 receptors/cell, in comparison to adult and old erythrocytes with 1-4 receptors/cell. The treatment of mouse erythrocytes with rHuEpo resulted in time- and dose-dependent up-regulation of NO production, mediated via activation of PI3K/Akt pathway and RBC-eNOS phosphorylation at Ser-1177. Along with the stimulation of *de novo* NO production, Epo affected the distribution of NO-metabolites between the cells and the medium. Finally, when erythrocytes were incubated in L-arginine-free medium, rHuEpo treatment resulted in upregulation of superoxide radical production with concomitant shifting of the cellular redox state towards more oxidized. Epo-induced changes in erythrocyte redox potential were absent in erythrocytes from eNOS deficient mice.

Key words

red blood cells, PI3K/Akt pathway, NO production, redox state

INTRODUCTION

Erythropoietin (Epo) is a major regulator of the red blood cell production, widely used in clinics to treat anemia (21, 44). Along with its erythropoietic properties, Epo nowadays is considered as a pleiotropic cytoprotective factor (22, 26). Binding of Epo to its receptor (EpoR) activates several survival signaling cascades including PI3K/Akt pathway in endothelial cells (4, 49) and cardiomyocytes (12). In both cell types studied, erythropoietin upregulates the expression of endothelial type nitric oxide synthase (eNOS) and directly enhances the enzyme activity via PI3K/Akt-mediated phosphorylation of serine 1177 (Ser-1177) (4, 12, 49). Recent reports suggested that Epo-induced regulation of NO production has a pivotal role in the cytoprotective effects of Epo (12, 18, 27, 49).

The presence of EpoR on erythrocyte membranes is disputable. The data found in the literature are controversial and the responses of mature RBCs to Epo treatment are poorly investigated. The expression of EpoR during the late erythroid development decreases exponentially and mature erythrocytes were claimed to virtually lack EpoR and to be Epo-insensitive (9, 57). However, specific binding of Epo to rat and human RBCs was reported (3, 42). Epo was shown to have an effect on glucose transport (3, 23), antioxidant defense system (13, 15), ion transport (5, 42) and rheological properties (24, 56) of mature red cells. At present, no data are available on the Epo-binding sites on erythrocyte membranes or their distribution within RBC population.

As a putative downstream target of Epo-induced signaling in red cells we have chosen eNOS (RBC-eNOS), which presence in human and mouse erythrocytes was recently reported (30). The activity of the RBC-eNOS is comparable to that observed in conventional endothelium-

derived eNOS (30). It is shown that insulin activates RBC-eNOS by phosphorylation of the enzyme at Ser-1177 via the PI3K/Akt pathway (30).

The aim of the present study was to characterize the binding of Epo to murine erythrocytes and the effect of physiological and therapeutic doses of Epo on RBC-eNOS. Our data indicate the presence of a single class Epo binding sites, similar in affinity and downstream targets to a classical Epo receptor (EpoR). We have shown that Epo-binding sites are not equally distributed within RBC population. Reticulocytes and young erythrocytes contain more Epo binding sites in comparison to adult and old cells. Epo treatment results in upregulation of erythrocyte NO production via activation of PI3K/Akt-signaling pathway and phosphorylation of RBC-eNOS at Ser-1177. Additionally we provide evidences for a direct link between the Epo-induced regulation of RBC-eNOS activity and the maintenance of the redox state in mouse red blood cells.

MATERIALS AND METHODS

All chemicals and kits used in this study were purchased from Sigma Aldrich, St. Louis, MO when not stated specially.

Animals and RBC preparation

In the present study we used male C57BL6 mice (wt), 12 to 20 weeks old and homozygous eNOS deficient mice ($eNOS^{-/-}$). The animals were raised in the sterile breeding facilities at the Institute of Physiology, University of Zurich. The mice were kept on a commercial diet in, approved by the Veterinary Department of Canton Zurich. All experiments were performed in accordance with the Swiss animal protection laws and institutional guidelines. Animals were euthanized with CO_2 and blood was collected immediately by cardiac puncture (0.8–1.2 ml) into heparinized syringes. The packed cells were washed three times in incubation medium (150 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 0.15 mM $MgCl_2$, 10 mM Tris-MOPS, 10 mM glucose, 10 mM sucrose, pH 7.4). Buffy coat and plasma were discarded during the washing steps. The homogeneity of erythrocyte suspensions was monitored by FACS analysis. After the buffy coat removal, the remaining non erythroid cells (CD45, CD16 or CD36 positive cells) were less than 0.02% of the total cell population (Supplemental Fig. S1). Washed erythrocytes were subsequently resuspended to a hematocrit of about 10% and immediately used for further experiments. All studies were performed in the presence of 3 mM L-arginine (L-Arg) unless stated otherwise.

Erythropoietin binding studies

Binding of erythropoietin to the mature murine erythrocytes was assessed by using ^{125}I-labeled recombinant human erythropoietin (^{125}I-Epo) with a molecular weight of 34,000 Da and a specific activity of 30 TBq/mmol (Amersham Biosciences, Freiburg, Germany) as

previously described (42). Briefly, washed erythrocytes were resuspended at 5% hematocrit in incubation medium containing 0.1% BSA. Aliquots from the cell suspension (100 µl) were incubated for 3 h at 4°C with different amounts of ^{125}I-Epo (24 pM - 494 pM). Thereafter non-bound radiolabeled Epo was removed by washing of the cells tree times with cold incubation medium, and cell-bound radioactivity was determined in a gamma counter (Kontron Gamma Counting System, Switzerland). Specific binding was determined by subtracting the values of ^{125}I-Epo in the presence of at least 100-fold excess of unlabeled recombinant human Epo (rHuEpo/Eprex, Janssen-Cilag AG, Baar/Schweiz) from those in the absence of non-labeled rHuEpo. To investigate the nature of the Epo-binding sites, we tested the maximal specific binding of iodinated Epo (240 pM ^{125}I-Epo, ± excess of non-labeled rHuEpo) in cells pre-treated for 2 h with antibody (dilution 1:50) raised against erythropoietin receptor (EpoR) (M-20, Santa Cruz Biotechnology, Heidelberg, Germany). Finally, to determine the distribution of Epo-binding sites within erythrocyte population, mouse RBCs were incubated with 240 pM ^{125}I-Epo for 2 h and were afterwards separated by density gradient centrifugation as described further in the "Methods and materials" section. Cell-bound radioactivity from the separated cells was determined in a gamma counter or visualized autoradiographycally on an x-ray film.

Density separation of mouse RBCs

In order to divide mouse erythrocytes according to their age, RBCs were separated on continuous Percoll density gradient (Percoll Plus, GE Healthcare, Freiburg, Germany) as described previously (37). The cells were divided into 3 fractions: reticulocytes and young RBCs (low cell density), adult (medium cell density) and old (high cell density) erythrocytes. The age of the different erythrocyte fractions was confirmed by the ratio of band 4.1a/4.1b as described elsewhere (5). The quality of the cell fractionation and the homogeneity of the RBC populations were verified by repeated density separation (Supplemental Fig. S2).

RBCs treatment

I - Characterization of molecular mechanism and kinetics of Epo effect on the RBC-eNOS function: erythrocytes from wt mice were incubated for 2 h at room temperature in the presence or absence of 1 U/ml rHuEpo. Aliquots were taken at 0, 30, 60, 120 min for western blot analysis and measurement of NO metabolites (NO_2^-/NO_3^-) in the medium and cells. As a negative control RBCs from $eNOS^{-/-}$ mice were subjected to the same experimental protocol and NO_2^-/NO_3^- levels in the medium and cells were estimated. In additional experiments, erythrocytes from wt and $eNOS^{-/-}$ mice were incubated for 2 h in the presence or absence of L-arginine and the levels of non-protein thiols were measured.

II - Dose-response of Epo on the RBC-eNOS function: wt RBC were incubated with 0, 1, 10, 50, 100 U/ml rHuEpo for 30 min at room temperature and RBC-eNOS activity was estimated.

III - Phosphatidylinositol-3-kinase-, PKB- and EpoR antibody- sensitivity, and L-NMA dependence of Epo-induced NO production: wt RBCs were pretreated for 30 min with either 1 μM wortmannin [Phosphatidylinositol-3-kinase (PI3K) blocker], 150 μM A6730 [PKB (Akt) inhibitor], EpoR antibody (M-20; dilution 1:10) or 3 mM N^G-Monomethyl-L-arginine (L-NMA). After the incubation, RBCs were treated with 10 U/ml rHuEpo for 1 h and activity of RBC-eNOS was measured.

Erythrocyte ghost preparation

After treatment, erythrocytes were centrifuged at 4,000 g for 5 min at 4^oC. Packed RBCs (about 500 μl) were hemolyzed in 2 ml of ice-cold lysis buffer (10 mM TRIS/HCl (1 mM EDTA, 10 μg/ml pepstatin A, 10 μg/ml leupeptin, 5 μg/ml aprotinin, 0.1 mM PMSF, 10 mM Na-pyrophosphate, 10 mM NaF, pH 7.4). Membranes were pelleted at 47,000 *g* for 20 min at 4°C (Sorvall centrifuge RC-5B; rotor SS-34; Thermo Electron Corp., Franklin, MA). The

supernatant was used as cytosolic extract and membranes were solubilized in lysis buffer containing 0.5% deoxycholate and 1% Triton X-100. The protein concentration of the samples was determined with BCA Protein Assay (Pierce; Rockford, Ill) with BSA as a standard.

Western blot

The proteins from both cytosolic and membrane fractions were separated by 7.5% or 10% SDS-PAGE (500 µg protein per lane), and transferred to Protan BA83 nitrocellulose membranes (Schleicher und Schuell, Dassel, Germany). Protein transfer was controlled by Ponceau red staining. Membranes were blocked for 1 h at room temperature and incubated overnight at 4^{o}C with primary antibodies against phospho-eNOS (Ser-1177; Cell Signaling Technology, Danvers, MA) or phospho-Akt/PKBα (Thr^{308}; Upstate, Lake Placid, NY). Staining with antibody against total actin was used as a loading control. After washing, membranes were incubated for 1 h at room temperature with the corresponding horseradish peroxidase-conjugated secondary antibodies (anti-mouse/anti-rabbit; Jackson ImmunoResearch Laboratories, West Grove, PA). The enhanced chemiluminescence detection Western blotting system was used for signal visualization.

Evaluation of RBC-eNOS activity

One of the widely used markers for NO production in biological systems are nitrite/nitrate (NO_2^-/NO_3^-) levels since they are stable oxidation products of NO (29). In our study, we measured NO_2^- in the incubation medium and RBCs using tri-iodide-based chemiluminescence assay described in details elsewhere (19). Briefly, an aliquot of 200 µl from the RBC suspensions was taken at given times and centrifuged for 1.5 min at 13,200 g. 170 µl of the medium was immediately separated from the packed cells and placed on ice until the assay was performed. Erythrocytes were reconstituted with 170 µl fresh, cold

medium and lysed with 25 µl of nitrite preservation solution containing 800 mM $K_3Fe(CN)_6$, 100 mM *N*-ethylmaleimide (NEM), 10% Nonidet-40. The samples were deproteinized by addition of 125 µl methanol (100%) and centrifuged for 10 min at 13,200g. 50 µl from the incubation medium or supernatant from the cell lysates were injected in the pre-heated (65^{o}C) reaction chamber containing acidic tri-iodide (I_3^-) reagent. The reagent was prepared fresh before the measurements by mixing 1.65 g KI, 0.57 g I_2 , 15 ml ddH_2O and 200 ml glacial CH_3COOH. The reaction chamber was purged with helium and released NO was detected using ECO-Medics CLD-88 analyzer (ECO MEDICS, Durnten, Switzerland). The signal was processed using PowerChrom 280 system (eDAQ Pty Ltd; Spechbach, Germany). In order to measure nitrate in the samples, we reduced the NO_3^- to NO_2^- using cadmium/copper based reduction kit NITRALYZER™-II (World Precision Instruments, Sarasota, Fl). The assay was performed according to the manufacturer instructions. After the reduction, NO_2^- was measured as described above. Nitrate levels were estimated by the subtraction of NO_2^- levels before the reduction from those obtained after the conversion of NO_3^- to NO_2^-.

Measurements of non-protein thiols

The amount of cellular glutathione (GSH) and oxidized glutathione (GSSG) was assayed in erythrocytes as described previously (6). Briefly, samples from RBC suspensions were mixed 1:10 with deproteinizing solution containing 1.67 g glacial metaphosphoric acid, 0.2 g Na_2EDTA, 30 g NaCl, 100 ml ddH_2O. After centrifugation, GSH concentration was determined in supernatants using 5,5'-dithiobis(2-nitrobenzoic acid) (Ellman's reagent). Optical density of the colored complex was measured photometrically at 412 nm. Simultaneously, aliquots from the same samples were incubated in the presence of glutathione reductase and NADPH for reduction of GSSG to GSH, and total glutathione levels (GSH+GSSG) were determined. The half-cell reduction potential (E_{hc}) was calculated according to Schafer and Buettner (50).

Measurements of superoxide anion production

To evaluate the influence of L-arginine deprivation and Epo on generation of superoxide anions ($*O_2^-$) by erythrocytes, we used chemiluminescence Superoxide Anion Assay Kit (CS1000). $5x10^6$ erythrocytes were incubated for 2 h in the presence or absence of L-Arg, or in the presence of 1 U/ml rHuEpo without eNOS substrate in the medium. The assay was performed according to the producer's guideline.

Statistical Analysis

All data are based on at least 6 experiments and are presented as mean ± SEM. The comparison between the experimental groups was performed using ANOVA and two-tailed Student's t-test for unpaired samples (GraphPad Instat.V3.05). The level of statistical significance was set at $P < 0.05$.

RESULTS

Epo binding to murine erythrocytes

Treatment of wt mouse erythrocytes with radiolabeled Epo (^{125}I-Epo) in the presence and in the absence of excessive amounts of non-radioactive Epo, revealed a specific binding of erythropoietin to the RBC membranes (Fig. 1*A*). The equilibrium binding isotherm (Fig. 1*B*) and scatchard analysis (Fig. 1*C*) indicated the presence of single type Epo-binding site with high affinity to Epo (K_D = 58.3 ± 11.1 pmol/l). We further performed separation of mouse erythrocytes pretreated with ^{125}I-Epo, according to their age. It is known that RBC density increase with cell aging (37). Using Percoll density gradient, RBCs were divided into tree major fractions: reticulocytes + young RBCs (low density), adult erythrocytes (medium density) and old cells (high density), as shown in Fig. 2*A*. The relative age differences between the RBC fractions were confirmed by the band 4.1a:4.1b ratio (Fig. 2, *B* and *C*), which increase with the cell ageing (54). Our data revealed unequal ^{125}I-Epo binding to the cells of the different erythrocyte fractions. Reticulocytes and young erythrocytes, representing 2.24 ± 0.14% of the RBC population (Fig. 2*D*) have markedly higher ability to bind ^{125}I-Epo (Fig. 3*A*). The number of Epo-binding sites per cell was respectively 105 ± 8 for low density, 4 ± 1 for medium density and 2 ± 1 for high density RBCs. The results obtained from the gamma counter were confirmed autoradiographycally. After the separation of mouse erythrocytes, pretreated with radioactive Epo, x-ray film was placed next to the centrifuge tube for about 6 hours. A specific band on the level of low density erythrocyte fraction was observed (Fig. 3*B*). Unfortunately no bands were detected on the film from the adult and old erythrocyte populations, most probably due to the weaker and diffuse signal. Finally, the pretreatment of the total RBC population with an antibody against Epo-R (M-20, dilution 1:100), reduced the specific ^{125}I-Epo binding from 427.5 ± 77.6 to 47.11 ± 136.1 DPM/100 µl

RBCs. These results suggest that murine erythrocytes specifically bind erythropoietin, and that its binding sites are recognized by EpoR-specific antibodies.

Epo triggers activation of the Akt and eNOS phosphorylation

We have further monitored the activity of the PI3K/Akt cascade known as a common EpoR-sensitive signaling pathway (21, 22, 26, 44). Western blot analysis revealed an increased phosphorylation of Akt in the cytosolic extracts from RBCs treated with 1 U/ml rHuEpo compared to the Epo-free controls (Fig. 4*A*). The phosphorylation peaked in the cells exposed to Epo for 30 min and decreased thereafter. The activation of Akt was followed by an increase in the phosphorylation of eNOS, one of its downstream targets. As shown in Fig. 4*B*, exposure of RBCs to 1 U/ml rHuEpo caused phosphorylation of the RBC-eNOS at Ser-1177. Upregulation of Ser-1177 phosphorylation could be detected in the membrane fractions already 30 min after cytokine administration reaching its maximum within 1 h of Epo treatment. Phosphorylation of eNOS at Ser-1177 is known to activate the enzyme (40). Therefore, we followed the kinetics and dose-dependence of Epo action on the NO production in murine erythrocytes

Kinetics of Epo effect on RBC-eNOS activity

We used NO_2^-/NO_3^- levels in the cells and incubation medium as markers of NO production (29) by erythrocytes, treated with various doses of rHuEpo for various time periods. The exposure of mouse erythrocytes to 1 U/ml rHuEpo triggered upregulation of NO production. The resulting nitrite was either removed from the cells or rapidly oxidized to nitrate when in the cytosol. The rate of nitrite accumulation in the incubation medium was doubled in the presence of 1 U/ml rHuEpo (Fig. 5*A*; 2.78 ± 0.52 compared to 1.35 ± 0.31 μmol/h*l_{cells} in the Epo-free control). Whereas intracellular NO_2^- levels remained virtually unchanged (Fig. 5*B*), NO_3^- content increased profoundly in the Epo-treated erythrocytes with new steady-state

levels twice exceeding the basal nitrate content (Fig. 5*D*). Unfortunately, nitrate accumulation in the incubation medium could not be assessed due to the high basal level of this anion in the incubation solution (Fig. 5*C*). The obtained results suggest that treatment of mouse erythrocytes with 1 U/ml rHuEpo caused a two-fold increase in the RBC-eNOS activity that could be detected already 30 min after the cytokine administration. Therefore, 30-60 min time frame was chosen for the assessment of the dose-response of Epo action on the eNOS function.

Dose-dependence of Epo effect on RBC-eNOS activity

The concentration of Epo (1 U/ml, rHuEpo) that we adhered when studying kinetics of Epo-induced RBC-eNOS activation, reflects the endogenous cytokine plasma levels after physiological stimulation by hypoxia, or decreased blood hemoglobin (1, 21, 26). Therapeutic Epo doses used in the clinics however vary between 150 and 40,000 U/kg (21, 26, 44). Thus, Epo plasma levels in patients reach 10-100 U/ml. To characterize the effects of physiological and therapeutic doses of Epo on the RBC-eNOS activity, we incubated wt erythrocytes with increasing amount of rHuEpo ranging between 1 and 100 U/ml.

Epo-induced changes of nitrite production over 30 min of incubation were dose-dependent, with a peak at 10 U/ml rHuEpo (Fig. 6*A*). When higher Epo doses were applied (50-100 U/ml), nitrite levels were somewhat lower than those at 10 U/ml. Of note, similar bi-phasic dose-dependent induction of NO production by Epo was reported in endothelial cell lines (4).

30 min of incubation were not sufficient to detect significant Epo-induced changes in RBC NO_2^- levels (data not shown). However, increase in the duration of rHuEpo treatment (1-100 U/ml) up to 1 h revealed Epo-induced accumulation of NO_2^- in the cytosol, with nitrite production exceeding its transport out of the cells and oxidation rate (Fig. 6*B*). In contrast,

NO_3^- accumulation in RBCs treated with the same doses of Epo was observed already after 30 min of incubation (Fig. 6*C*). Similar to the results obtained for NO_2^- levels in the medium, Epo-induced NO_3^- accumulation in the cells was bi-phasic with a maximum at 10 U/ml rHuEpo (compare Fig. 6, *A* and *C*).

Lack of Epo effect on NO_2^-/NO_3^- levels in RBCs from $eNOS^{-/-}$ mice

To confirm the specific effect of erythropoietin on RBC-eNOS, we studied the Epo-induced responses on NO_2^-/NO_3^- levels in erythrocytes obtained from $eNOS^{-/-}$ mice. Basal nitrite levels in the medium during the incubation of eNOS-deficient RBCs were significantly lower when compared to the values obtained with wt erythrocytes (Fig. 7*A*; 0.599 ± 0.33 μmol/120 min*l_{cells} and 1.8 ± 0.29 μmol/120 min*l_{cells} respectively, $P < 0.05$). The treatment of $eNOS^{-/-}$ RBCs with 10 U/ml rHuEpo failed to affect the NO_2^- levels in the medium (Fig. 7*A*) and erythrocytes (Fig. 7*B*), and the cellular NO_3^- content (Fig. 7*C*). These results strongly suggest that the observed Epo-induced changes of NO_2^-/NO_3^- levels in the wt erythrocytes and incubation medium are due to the specific action of Epo on the endothelial type nitric oxide synthase present in the murine RBCs.

White blood cells do not contribute to Epo-induced regulation of RBC-eNOS

In the next set of experiments we assessed the possible influence of white blood cells on Epo induced regulation of RBC-eNOS. The presence of buffy coat did not affect Epo-induced (10 U/ml rHuEpo) increase of NO_2^- levels in the incubation medium (Δ NO_2^-, 0.67 ± 0.13 μmol/h*l_{RBC} with and 0.69 ± 0.18 μmol/h*l_{RBC} without buffy coat) or erythrocyte NO_3^- content (Δ NO_3^-, 387.6 ± 86.5 μmol/h*l_{RBC} with and 370 ± 49.3 μmol/h*l_{RBC} without buffy coat). Obtained results confirm the specific, direct and acute modulatory effect of Epo on the erythrocyte NO production.

Blocking of Epo-induced activation of RBC-eNOS

To further characterize the mechanism behind Epo-induced activation, we blocked different components of the putative signaling pathway and monitored the NO_2^-/NO_3^- levels in the presence or absence of Epo. In agreement with the data obtained for the Epo-induced phosphorylation of Akt and RBC-eNOS, the activation of NO production by Epo was completely abolished by the pre-treatment of RBCs with 1 μM PI3K inhibitor wortmannin, 150 μM Akt blocker A6730 or EpoR antibody (M-20, dilution 1:100) (Fig. 8, *A* and *B*).

Effect of Epo on the distribution of NO_2^-/NO_3^-

RBCs are able to store NO and its derivatives, releasing them into the circulation upon deoxygenation (28, 46). In the next set of experiments, we tested whether Epo may affect the redistribution of NO_2^-/NO_3^- between the cells and the medium, when *de novo* NO_2^- production is blocked by L-NMA. The administration of eNOS blocker completely abolished the Epo-induced increase of NO_3^- in RBCs (Fig. 9*A*). However, NO_2^- accumulation in the medium triggered by Epo was only partially suppressed and was significantly exceeding that in the untreated RBCs (Fig. 9*B*). The latter was followed, by a concomitant NO_2^- depletion in Epo-treated erythrocytes (Fig 9*B*). This observation implies that Epo facilitates NO_2^- release into the incubation medium simultaneously with stimulation of *de novo* NO synthesis. Epo-induced activation of NO_2^- redistribution between the cells and the medium seems to be independent from its action on RBC-eNOS activity.

L-Arg availability and Epo-treatment modulate the redox state of RBCs

Under certain conditions, such as substrate deficiency, eNOS produces superoxide anions ($^*O_2^-$) instead of NO (14, 41), causing oxidative stress. We tested if Epo may induce an oxidative stress in red cells deprived of L-arginine (L-Arg). The effect of Epo on NO production and cellular redox state in RBCs incubated in L-Arg-containing and L-Arg-free

medium was monitored. Epo failed to increase NO_2^-/NO_3^- levels in the erythrocytes and incubation medium in the absence of L-Arg (data not shown). Moreover, the omission of L-Arg triggered a significant upregulation of $*O_2^-$ generation by the RBCs, which was further facilitated by Epo (Fig. 10*A*).

Both, NO and $*O_2^-$ generation contribute substantially to the maintenance of the cellular redox state. We measured intracellular reduced (GSH) and oxidized (GSSG) glutathione levels after 2 h of incubation with 1-100 U/ml rHuEpo in the presence and absence of L-Arg and expressed the data as a half-cell redox potential for the GSH:GSSG couple (50). Incubation of the RBCs in L-Arg-free medium shifted the half-cell reduction potential to more oxidized (Fig. 10*B*). Treatment with 1 U/ml rHuEpo further facilitated GSH oxidation (Fig. 10*B*). In L-Arg-containing medium the redox potential was preserved in cells incubated with 1 U/ml rHuEpo for at least 2 h. Acute treatment of RBCs with a therapeutic dose of 100 U/ml rHuEpo in the presence of L-Arg caused a statistically significant decrease in the half-cell reduction potential, indicating a shift to more reduced state. Our data suggest that the influence of Epo-induced regulation of RBC-eNOS on the cellular redox state depends on the presence of L-Arg. Depending on the L-Arg availability Epo may play a dual role as pro- or antioxidant.

Finally, we tested if Epo and L-Arg will affect cellular redox state of the $eNOS^{-/-}$ erythrocytes. As expected, the availability of the eNOS substrate was without an effect on the half cell redox potential of eNOS deficient RBCs (-291.7 ± 5.6 mV in the presence and -290.2 ± 3.3 mV in the absence of 3 mM L-Arg in the medium). Moreover, 100 U/ ml rHuEpo failed to affect the cellular redox state in the presence or absence of L-Arg (-287.6 ± 13.4 in the absence and -292.4 ± 8.5 in the presence of 3 mM L-Arg in the medium).

DISCUSSION

Our study is the first to characterize the interaction of Epo with murine red blood cells. The obtained data imply that eNOS is one of the targets of Epo action in mouse erythrocytes. By activating RBC-eNOS, Epo may have a profound effect on a number of cellular properties of which maintenance of the redox potential is of key importance.

Characteristics of Epo binding sites on murine erythrocytes

We have detected the presence of Epo binding sites on murine erythrocyte membrane. Skatchard analysis of the data on the dose-response of ^{125}I-Epo binding (Fig. 1, *B* and *C*) reveals a single binding site class with a $K_D = 58.3 \pm 11.1$ pmol/l, a value within the range of affinity of classical Epo receptor (EpoR) to erythropoietin reported in the literature (30 to 330 pmol/l) (9, 42, 52, 57). The wide scatter of the KD values reveals a broad tissue and species-specific variability of the EpoR properties. Interaction of Epo with the erythrocyte membrane could be abolished by pretreatment of the cells with an antibody against mouse EpoR, which was claimed to target the receptor specifically (20). Furthermore, Epo binding to the cells activated PI3K/Akt signaling cascade (Figs. 4 and 7), a well-characterized downstream signaling pathway of a common EpoR in erythroid precursor cells (21, 22, 26, 44). Taken together, these observations suggest that Epo interacts with a common Epo receptor present on erythrocyte membranes. The low number of receptors as well as the absence of reliable highly specific antibodies makes the direct molecular identification technically demanding.

Previous studies using ^{125}I-Epo to track Epo binding to human erythrocytes reported the presence of 5-6 Epo binding sites per cell, based on the assumption that the number of specific Epo binding sites is equal for each erythrocyte (42). If the same assumption is applied to our data, similar numbers may be obtained (5-6 Epo binding sites per mouse erythrocyte).

However, the number of binding sites per cell varies between 100 and 2 binding site per cell, depending on the cell age (Fig. 3). The marked decrease in Epo-binding capability with cell ageing is most likely achieved as Epo receptor is released in vesicles during cell maturation (36). The results suggest that all erythrocytes have a potential to be Epo-sensitive, but the degree and significance of the cellular response should be age-dependent and requires further clarification. Unfortunately, no functional studies can be performed on the Epo-induced NO production in sub-fractions obtained by Percoll density gradient centrifugation, since the contact with the Percoll rendered the cells Epo-insensitive (data not shown). Since released, NO may diffuse affecting all the neighboring cells when in blood stream. Therefore, the Epo effects on NO production that we have observed in non-separated red cells population are physiologically relevant even when generated by relatively few cells. However, the magnitude of the Epo-induced response will depend critically on the number of young cells

Epo-induced regulation of NO production and extracellular NO_2^- levels

We have shown that Epo triggers an increase in the activity of the eNOS in mouse erythrocytes by promoting phosphorylation of the enzyme at Ser-1177 by Akt (Fig. 4). This adds erythropoietin to the list of physiological regulators of the RBC-eNOS along with insulin, acting through the same signaling cascade (30). RBC-born nitric oxide, in addition to the up-regulation of NO production by endothelial cells and cardiac myocytes, would facilitate the cytoprotective effect of Epo (4, 12, 39, 49).

Since Epo is strongly considered as a potential cardioprotective agent (7, 12, 22, 26), the dose-dependence of the NO release is of particular importance. The acute up-regulation of NO production in RBCs could be detected after administration of 1 U/ml rHuEpo (Fig. 5). This Epo concentration exceeds the one found in normoxic human or mouse blood (0.001 to 0.027 U/ml) (21, 26, 44) by several orders of magnitude, but might be reached *in vivo* in

response to hypoxia or anemia (1-10 U/ml) (1). We cannot exclude that the long-term effects of Epo on RBC-eNOS may be observed at much lower doses, than those used in our study. Epo doses used in the clinics are however in the range between 150 and 40,000 U/kg corresponding to 10-100 U/ml in plasma (21, 26, 44). Our data indicate that the increase in Epo dose above 10 U/ml does not cause further increase in the NO production (Fig. 6). In fact the Epo-induced regulation of RBC-eNOS was bi-phasic and resembled that reported in the endothelium (4).

The role of Epo in controlling the cellular redox state

eNOS and its activity are involved into the regulation of the cellular redox state. Under conditions of substrate or cofactor deficiency and in response to atherogenic stimuli, eNOS itself generates superoxide anions rather than NO (14, 41). We have shown that this is the case when Epo-induced activation of RBC-eNOS occurs in L-Arg-free medium (Fig. 10). The obtained results imply that Epo can be pro- or anti-oxidant depending on the conditions. Oxidative stress is one of the factors triggering RBCs senescence (34, 35). Thus, Epo may accelerate or attenuate senescence depending on the L-Arg availability (2). Of note, our experiments were performed with RBCs equilibrated with 20% oxygen (O_2) and thus fully O_2-saturated. In the organism, where O_2 saturation varies from almost 100% in lung arterioles to about 10-20% in venous blood, $*O_2^-$ production may be reduced compared to that we report here. However, in pathological conditions Epo-induced changes in cellular redox state may become significant.

Our results may have particularly important consequences for the pathophysiology of rHuEpo therapy. Increased oxidative stress is a feature characteristic in patients with chronic renal failure undergoing hemodialysis (HD) (25, 33, 45). Several publications reported that Epo-treatment of uremic patients on chronic HD is accompanied by further increase of oxidative

stress and requires co-application of antioxidants (17, 32, 43). A possible cause for these observations could be L-Arg deficiency and uncoupling of eNOS activity via inhibition of L-Arg uptake (10, 38, 59) or changes in extracellular L-Arg content (10, 11, 58). Our data suggest that co-application of Epo and L-Arg would help to avoid probable side effects of Epo treatment. However, since erythrocyte properties are species dependent, further experiments are necessary for characterization of the effects of Epo on human RBCs in physiology and pathophysiology.

Physiological relevance of Epo-induced activation of the eNOS in red cells

Our findings along with the previous reports raise an important question that remains to be answered: what is the role of RBC-born NO and Epo-induced regulation of RBC-eNOS activity. When Epo plasma levels increase under hypoxic conditions, together with an augment in RBC mass, Epo-induced NO production could improve tissue's blood supply due to NO-mediated vasodilatation (16, 28, 51). Further estimation of the relative contribution of RBC-eNOS activity to the total NO pool remain to be characterized, however increasing the local NO concentration would prevent the Hb consumption of NO derived from other sources. Thus Epo-induced upregulation of RBC NO production may be considered as an adaptive strategy to cope with the reduced tissue oxygenation. Additionally, nitric oxide production affects RBC membrane fluidity and deformability (8, 30) thus improving capillary perfusion and reducing sheer stress (56). In hypoxic areas accumulated plasma NO_2^- can be reduced to NO and released by RBCs to further improve O_2 delivery (16, 19, 28, 51). Finally, oxygen release from Hb in hypoxic conditions is also affected, as NO reduces the oxygen affinity of Hb, enhancing the oxygen transfer from erythrocytes to the tissues (31, 53).

Another interesting aspect that draws attention is the unequal distribution of Epo binding sites within RBC population and its role for the cells. We found that reticulocytes and young

erythrocytes contain much more Epo receptors in comparison to the adult and old ones, suggesting that young RBCs are more sensitive to changes in Epo plasma levels. In the last years several papers described a process of selective lysis of relatively young erythrocytes called neocytolysis (2, 48, 55). Neocytolysis occurs in individuals acclimatized to high altitude on descent to sea level or astronauts ascending in to space and is considered as a physiological process for down-regulation of excessive red-cell mass (2). The exact mechanism of neocytolysis is still unclear, but it was found that it is accompanied by decrease of Epo plasma levels and selective removal of relatively young erythrocytes, generated over the previous 10-11 days (47). Our data suggest that the number of Epo-binding sites may be the key factor for selecting and survival of the different erythrocyte fractions. The greater number of Epo-binding sites on the young RBCs could explain their preferential removal when Epo plasma levels drop below a certain threshold.

In summary, our findings indicate that mouse erythrocytes possess classical Epo receptors, which number per cell depends on the RBC age. Erythropoietin activates RBC-eNOS and regulates the distribution of NO-bioactive species between the red cells and the medium. Furthermore, the Epo-induced regulation of eNOS activity affects redox state of the RBCs by generation of NO or $*O_2^-$ depending on L-Arg bioavailability. Pro-oxidative potential of rHuEpo should be seriously considered when using this drug in clinics.

ACKNOWLEDGMENTS

We thank Dr. Hans U. Lutz, Institute of Biochemistry, ETH Zurich for his helpful comments and suggestions.

This work was supported by Swiss National Science Foundation (3100B0-112449 to AB) and Zurich Center for Integrative Human Physiology (ZIHP).

REFERENCES

1. **Abbrecht PH and Littell JK.** Plasma erythropoietin in men and mice during acclimatization to different altitudes. *J Appl Physiol* 32: 54-58, 1972.
2. **Alfrey CP, Rice L, Udden MM, and Driscoll TB.** Neocytolysis: physiological down-regulator of red-cell mass. *Lancet* 349: 1389-1390, 1997.
3. **Baciu I, Ivanof L, Pavel T, Marina C, Zirbo M, and Zdrenghea C.** Erythropoietin binding to the red cell membranes. *Physiologie* 22: 227-231, 1985.
4. **Beleslin-Cokic BB, Cokic VP, Yu X, Weksler BB, Schechter AN, and Noguchi CT.** Erythropoietin and hypoxia stimulate erythropoietin receptor and nitric oxide production by endothelial cells. *Blood* 104: 2073-2080, 2004.
5. **Bogdanova A, Mihov D, Lutz H, Saam B, Gassmann M, and Vogel J.** Enhanced erythro-phagocytosis in polycythemic mice overexpressing erythropoietin. *Blood* 110: 762-769, 2007.
6. **Bogdanova AY, Ogunshola OO, Bauer C, and Gassmann M.** Pivotal role of reduced glutathione in oxygen-induced regulation of the Na(+)/K(+) pump in mouse erythrocyte membranes. *The Journal of membrane biology* 195: 33-42, 2003.
7. **Bogoyevitch MA.** An update on the cardiac effects of erythropoietin cardioprotection by erythropoietin and the lessons learnt from studies in neuroprotection. *Cardiovasc Res* 63: 208-216, 2004.
8. **Bor-Kucukatay M, Wenby RB, Meiselman HJ, and Baskurt OK.** Effects of nitric oxide on red blood cell deformability. *Am J Physiol Heart Circ Physiol* 284: H1577-1584, 2003.
9. **Broudy VC, Lin N, Brice M, Nakamoto B, and Papayannopoulou T.** Erythropoietin receptor characteristics on primary human erythroid cells. *Blood* 77: 2583-2590, 1991.
10. **Brunini T, Resende A, Moss M, Soares de Moura R, and Mendes Ribeiro A.** L-Arginine Availability as a Pathological Mechanism in Essential Hypertension, Chronic Renal and Heart Failure. *Vascular Disease Prevention* 2: 37-51, 2005.
11. **Brunini TM, Roberts NB, Yaqoob MM, Ellory JC, Mann GE, and Mendes Ribeiro AC.** Activation of L-arginine transport in undialysed chronic renal failure and continuous ambulatory peritoneal dialysis patients. *Clinical and experimental pharmacology & physiology* 33: 114-118, 2006.
12. **Burger D, Lei M, Geoghegan-Morphet N, Lu X, Xenocostas A, and Feng Q.** Erythropoietin protects cardiomyocytes from apoptosis via up-regulation of endothelial nitric oxide synthase. *Cardiovasc Res* 72: 51-59, 2006.
13. **Chakraborty M, Ghosal J, Biswas T, and Datta AG.** Effect of erythropoietin on membrane lipid peroxidation, superoxide dismutase, catalase, and glutathione peroxidase of rat RBC. *Biochemical medicine and metabolic biology* 40: 8-18, 1988.
14. **Channon KM and Guzik TJ.** Mechanisms of superoxide production in human blood vessels: relationship to endothelial dysfunction, clinical and genetic risk factors. *J Physiol Pharmacol* 53: 515-524, 2002.
15. **Chattopadhyay A, Choudhury TD, Bandyopadhyay D, and Datta AG.** Protective effect of erythropoietin on the oxidative damage of erythrocyte membrane by hydroxyl radical. *Biochemical pharmacology* 59: 419-425, 2000.
16. **Crawford JH, Isbell TS, Huang Z, Shiva S, Chacko BK, Schechter AN, Darley-Usmar VM, Kerby JD, Lang JD, Jr., Kraus D, Ho C, Gladwin MT, and Patel RP.** Hypoxia, red blood cells, and nitrite regulate NO-dependent hypoxic vasodilation. *Blood* 107: 566-574, 2006.

17. **Cristol JP, Bosc JY, Badiou S, Leblanc M, Lorrho R, Descomps B, and Canaud B.** Erythropoietin and oxidative stress in haemodialysis: beneficial effects of vitamin E supplementation. *Nephrol Dial Transplant* 12: 2312-2317, 1997.
18. **d'Uscio LV, Smith LA, Santhanam AV, Richardson D, Nath KA, and Katusic ZS.** Essential Role of Endothelial Nitric Oxide Synthase in Vascular Effects of Erythropoietin. *Hypertension* 49: 1142-1148, 2007.
19. **Dejam A, Hunter CJ, Pelletier MM, Hsu LL, Machado RF, Shiva S, Power GG, Kelm M, Gladwin MT, and Schechter AN.** Erythrocytes are the major intravascular storage sites of nitrite in human blood. *Blood* 106: 734-739, 2005.
20. **Elliott S, Busse L, Bass MB, Lu H, Sarosi I, Sinclair AM, Spahr C, Um M, Van G, and Begley CG.** Anti-Epo receptor antibodies do not predict Epo receptor expression. *Blood* 107: 1892-1895, 2006.
21. **Fisher JW.** Erythropoietin: physiology and pharmacology update. *Experimental biology and medicine (Maywood, NJ* 228: 1-14, 2003.
22. **Ghezzi P and Brines M.** Erythropoietin as an antiapoptotic, tissue-protective cytokine. *Cell death and differentiation* 11 Suppl 1: S37-44, 2004.
23. **Ghosal J, Chakraborty M, Biswas T, Ganguly CK, and Datta AG.** Effect of erythropoietin on the glucose transport of rat erythrocytes and bone marrow cells. *Biochemical medicine and metabolic biology* 38: 134-141, 1987.
24. **Hassan K, Roguin N, Kaganov Y, Hasan S, and Kristal B.** Effect of erythropoietin therapy on red cells filterability and left ventricular mass in predialysis patients. *Renal failure* 27: 177-182, 2005.
25. **Jacob HS, Eaton JW, and Yawata Y.** Shortened red blood cell survival in uremic patients: beneficial and deleterious effects of dialysis. *Kidney international*: 139-143, 1975.
26. **Jelkmann W.** Erythropoietin after a century of research: younger than ever. *Eur J Haematol* 78: 183-205, 2007.
27. **Jie KE, Verhaar MC, Cramer M-JM, van der Putten K, Gaillard CAJM, Doevendans PA, Koomans HA, Joles JA, and Braam B.** Erythropoietin and the cardiorenal syndrome: cellular mechanisms on the cardiorenal connectors. *Am J Physiol Renal Physiol* 291: F932-944, 2006.
28. **Kim-Shapiro DB, Schechter AN, and Gladwin MT.** Unraveling the Reactions of Nitric Oxide, Nitrite, and Hemoglobin in Physiology and Therapeutics. *Arterioscler Thromb Vasc Biol* 26: 697-705, 2006.
29. **Kleinbongard P, Dejam A, Lauer T, Rassaf T, Schindler A, Picker O, Scheeren T, Gödecke A, Schrader J, Schulz R, Heusch G, Schaub GA, Bryan NS, Feelisch M, and Kelm M.** Plasma nitrite reflects constitutive nitric oxide synthase activity in mammals. *Free Radic Biol Med* 35: 790-796 2003.
30. **Kleinbongard P, Schulz R, Rassaf T, Lauer T, Dejam A, Jax T, Kumara I, Gharini P, Kabanova S, Ozuyaman B, Schnurch H-G, Godecke A, Weber A-A, Robenek M, Robenek H, Bloch W, Rosen P, and Kelm M.** Red blood cells express a functional endothelial nitric oxide synthase. *Blood* 107: 2943-2951, 2006.
31. **Kosaka H and Seiyama A.** Increased oxygen dissociation by nitric oxide from RBC. *Advances in experimental medicine and biology* 428: 349-354, 1997.
32. **Linde T, Sandhagen B, Danielson BG, and Wikstrom B.** Impaired erythrocyte fluidity during treatment of renal anaemia with erythropoietin. *Journal of internal medicine* 231: 601-606, 1992.
33. **Loughrey CM, Young IS, Lightbody JH, McMaster D, McNamee PT, and Trimble ER.** Oxidative stress in haemodialysis. *Qjm* 87: 679-683, 1994.
34. **Lutz HU.** Innate immune and non-immune mediators of erythrocyte clearance. *Cellular and molecular biology (Noisy-le-Grand, France)* 50: 107-116, 2004.

35. **Lutz HU, Fasler S, Stammler P, Bussolino F, and Arese P.** Naturally occurring anti-band 3 antibodies and complement in phagocytosis of oxidatively-stressed and in clearance of senescent red cells. *Blood cells* 14: 175-203, 1988.
36. **Lutz HU, Liu SC, and Palek J.** Release of spectrin-free vesicles from human erythrocytes during ATP depletion. I. Characterization of spectrin-free vesicles. *The Journal of cell biology* 73: 548-560, 1977.
37. **Lutz HU, Stammler P, Fasler S, Ingold M, and Fehr J.** Density separation of human red blood cells on self forming Percoll gradients: correlation with cell age. *Biochimica et biophysica acta* 1116: 1-10, 1992.
38. **Maarsingh H, de Boer J, Kauffman HF, Zaagsma J, and Meurs H.** Heparin normalizes allergen-induced nitric oxide deficiency and airway hyperresponsiveness. *British journal of pharmacology* 142: 1293-1299, 2004.
39. **Marzo F, Lavorgna A, Coluzzi G, Santucci E, Tarantino F, Rio T, Conti E, Autore C, Agati L, and Andreotti F.** Erythropoietin in heart and vessels: focus on transcription and signalling pathways. *Journal of thrombosis and thrombolysis*, 2008.
40. **Mounta P, Kempc B, and Powera D.** Regulation of endothelial and myocardial NO synthesis by multi-site eNOS phosphorylation *J Mol Cell Cardiol* 42: 271-279, 2006.
41. **Munzel T, Daiber A, Ullrich V, and Mulsch A.** Vascular Consequences of Endothelial Nitric Oxide Synthase Uncoupling for the Activity and Expression of the Soluble Guanylyl Cyclase and the cGMP-Dependent Protein Kinase. *Arterioscler Thromb Vasc Biol* 25: 1551-1557, 2005.
42. **Myssina S, Huber SM, Birka C, Lang PA, Lang KS, Friedrich B, Risler T, Wieder T, and Lang F.** Inhibition of erythrocyte cation channels by erythropoietin. *J Am Soc Nephrol* 14: 2750-2757, 2003.
43. **Nemeth I, Turi S, Haszon I, and Bereczki C.** Vitamin E alleviates the oxidative stress of erythropoietin in uremic children on hemodialysis. *Pediatric nephrology (Berlin, Germany)* 14: 13-17, 2000.
44. **Ng T, Marx G, Littlewood T, and Macdougall I.** Recombinant erythropoietin in clinical practice. *Postgrad Med J* 79: 367-376, 2003.
45. **Nguyen-Khoa T, Massy ZA, De Bandt JP, Kebede M, Salama L, Lambrey G, Witko-Sarsat V, Drueke TB, Lacour B, and Thevenin M.** Oxidative stress and haemodialysis: role of inflammation and duration of dialysis treatment. *Nephrol Dial Transplant* 16: 335-340, 2001.
46. **Pawloski JR, Hess DT, and Stamler JS.** Export by red blood cells of nitric oxide bioactivity. *Nature* 409: 622-626, 2001.
47. **Rice L, Ruiz W, Driscoll T, Whitley CE, Tapia R, Hachey DL, Gonzales GF, and Alfrey CP.** Neocytolysis on descent from altitude: a newly recognized mechanism for the control of red cell mass. *Annals of internal medicine* 134: 652-656, 2001.
48. **Risso A, Turello M, Biffoni F, and Antonutto G.** Red blood cell senescence and neocytolysis in humans after high altitude acclimatization. *Blood Cells, Molecules, and Diseases* 38: 83-92, 2007.
49. **Santhanam AVR and Katusic ZS.** Erythropoietin and cerebral vascular protection: role of nitric oxide. *Acta Pharmacologica Sinica* 27: 1389-1394, 2006.
50. **Schafer FQ and Buettner GR.** Redox environment of the cell as viewed through the redox state of the glutathione disulfide/glutathione couple. *Free radical biology & medicine* 30: 1191-1212, 2001.
51. **Singel DJ and Stamler JS.** Chemical physiology of blood flow regulation by red blood cells: the role of nitric oxide and S-nitrosohemoglobin. *Annu Rev Physiol* 67: 99-145, 2005.
52. **Spivak JL, Avedissian LS, Pierce JH, Williams D, Hankins WD, and Jensen RA.** Isolation of the full-length murine erythropoietin receptor using a baculovirus expression system. *Blood* 87: 926-937, 1996.

53. **Stepuro TL and Zinchuk VV.** Nitric oxide effect on the hemoglobin-oxygen affinity. *J Physiol Pharmacol* 57: 29-38, 2006.
54. **Suzuki T and Dale GL.** Membrane proteins in senescent erythrocytes. *The Biochemical journal* 257: 37-41, 1989.
55. **Trial J and Rice L.** Erythropoietin withdrawal leads to the destruction of young red cells at the endothelial-macrophage interface. *Current pharmaceutical design* 10: 183-190, 2004.
56. **Vogel J, Kiessling I, Heinicke K, Stallmach T, Ossent P, Vogel O, Aulmann M, Frietsch T, Schmid-Schonbein H, Kuschinsky W, and Gassmann M.** Transgenic mice overexpressing erythropoietin adapt to excessive erythrocytosis by regulating blood viscosity. *Blood* 102: 2278-2284, 2003.
57. **Wickrema A, Krantz S, Winkelmann J, and Bondurant M.** Differentiation and erythropoietin receptor gene expression in human erythroid progenitor cells *Blood* 80: 1940-1949, 1992.
58. **Wu G, Flynn NE, Flynn SP, Jolly CA, and Davis PK.** Dietary Protein or Arginine Deficiency Impairs Constitutive and Inducible Nitric Oxide Synthesis by Young Rats. *J Nutr* 129: 1347-1354, 1999.
59. **Yang Z and Kaye DM.** Endothelial dysfunction and impaired L-arginine transport in hypertension and genetically predisposed normotensive subjects. *Trends in cardiovascular medicine* 16: 118-124, 2006.

FIGURE LEGENDS

Fig. 1. Binding of ^{125}I-erythropoeitn to erythrocytes. *A*: Erythrocytes from wt mice were exposed to 24 pM or 240 pM of ^{125}I-erythropoietin (^{125}I-Epo; molecular weight, 34,000 Da; specific activity, 30 TBq/mmol) for 3 hours at 4^{o}C. The presence of excess (at least 100x) of non-labeled Epo reduced the binding (grey columns) compared to the samples treated only with radioactive Epo (black columns). *$P < 0.05$ compared with RBCs treated only with ^{125}I-Epo; n = 6; mean ± SEM. *B*: Specific binding of ^{125}I-Epo to the RBCs membranes in the presence of different amounts of labeled Epo. Erythrocytes were incubated for 3 hours at 4^{o}C. *C*: Scatchard analysis of the data from panel B.

Fig. 2. Separation of mouse erythrocytes according to the cell age. *A*: 500 µl of packed RBCs were mixed with 13.5 ml of 90% Percoll solution and centrifuged for 40 min at 35,000 g. Three fractions of red cells were harvested and membrane ghosts were isolated for evaluation of the cell age. *B*: Silver-stained SDS–polyacrylamide gel electrophoresis (PAGE) of erythrocyte membrane proteins. The part of the gel containing bands 3 through 4.2 is shown. *C*: Quantification of the band 4.1a to 4.1b ratio revealed straight correlation between erythrocyte age and cell density. *D*: Age-distribution within the erythrocyte population.

Fig. 3. Distribution of Epo-binding sites within RBC population. *A*: Binding of ^{125}I-Epo to the different RBC fractions, measured by gamma counter. *$P < 0.05$ compared to fraction-1 (reticulocytes + young RBCs), #$P < 0.05$ compared with fraction-2 (adult RBCs); n = 6; mean ± SEM. *B*: Autoradiography of density separated erythrocytes. The Percoll density gradient of ^{125}I-Epo treated RBCs is presented on the right with the corresponding x-ray image on the left.

Fig. 4. Erythropoietin triggers Akt and RBC-eNOS phosphorylation in mouse erythrocytes. Erythrocyte lysates from wt mice were divided in to cytosolic (cyt) and membrane (mem) fractions and proteins separated by SDS-PAGE. *A*: Western blot analysis revealed that Epo treatment resulted in increased phosphorylation of Akt. The signal was maximal at 30 min time point and gradually decreased thereafter, as binding was observed only in cytosolic fractions. *B*: Epo induced phosphorylation of RBC-eNOS at Ser-1177. A specific signal at 132 kDa was detected 30 min after Epo administration. Maximum in RBC-eNOS phosphorylation was observed after 1 h of incubation and than the signal decreased. Specific signal was observed predominantly in the membrane fractions.

Fig. 5. Kinetic of Epo effect on RBC-eNOS activity. RBC-eNOS activity was estimated by monitoring NO_2^-/NO_3^- levels in the incubation medium and cells. Erythrocytes from wt mice were incubated for 2 h in the presence (closed circles) or absence (open circles) of 1 U/ml rHuEpo. *A*: The accumulation rate and steady state of NO_2^- in the incubation medium were elevated by Epo. *B*: A transient depletion of basal NO_2^- cellular levels was observed. Epo treatment stabilized NO_2^- content of the erythrocytes. *C*: NO_3^- levels in the incubation medium did not change significantly during the incubation of the erythrocytes or after Epo administration. *D*: Epo treatment resulted in pronounced accumulation of NO_3^- in the RBCs. $*P < 0.05$ compared with control; n = 6; mean ± SEM.

Fig. 6. Dose dependence of Epo effect on RBC-eNOS activity. Erythrocytes from wt mice were incubated in the presence of 0-100 U/ml rHuEpo and NO_2^-/NO_3^- levels in the medium and cells were measured. *A*: 30 min were enough to detect dose dependent Epo-induced changes of NO_2^- levels in the incubation medium, with maximal response observed at 10 U/ml rHuEpo. *B*: For the detection of significant Epo effect on intracellular NO_2^- levels, RBCs were incubated for 1 h. The optimal Epo dose in this case was 50 U/ml. *C*: Epo

triggered dose dependant increase in cellular NO_3^- after 30 min of incubation. The optimal dose was again 10 U/ml rHuEpo. *$P < 0.05$ compared with untreated cells (white bars); n = 6; mean ± SEM.

Fig. 7. Epo does not affect NO_2^-/NO_3^- levels in erythrocytes from eNOS knockout ($eNOS^{-/-}$) mice. Erythrocytes from $eNOS^{-/-}$ mice were incubated for 2 h in the presence (closed triangles) or absence (open triangles) of 10 U/ml rHuEpo. The corresponding basal values obtained from wt erythrocytes (open circles) are included for comparison. *A*: The basal NO_2^- levels in the medium of eNOS erythrocytes were significantly low in comparison to wt. Epo administration did not cause any change in the extracellular nitrite levels. *B*: Epo treatment had no effect on the cellular NO_2^- content of $eNOS^{-/-}$ RBCs. *C*: Addition of rHuEpo to $eNOS^{-/-}$ erythrocytes failed to affect cytosolic NO_3^- levels. *$P < 0.05$ wt control compared with $eNOS^{-/-}$ control; n=6; mean ±SEM.

Fig. 8. Blocking of Epo-induced activation of RBC-eNOS. Wt RBCs were pretreated with either 1 μM wortmannin, 150 μM A6730 or Epo receptor antibody (M-20, dilution 1:100) for 30 min to block PI3K, Akt or Epo-binding to the cells. Further RBC suspensions were incubated with 10 U/ml rHuEpo for 1 h. Results are presented as Epo-induced changes of nitrite/nitrate levels (ΔNO_2^- / ΔNO_3^-) in the absence or presence of inhibitors. X-axis represents the basal NO_2^-/NO_3^- levels in the medium and RBCs in the absence of Epo. *A*: Epo-induced increase of cellular and extracellular NO_2^- levels was abolished by pretreatment of the RBCs with wortmannin, A6730 or M-20. *B*: Epo failed to affect NO_3^- levels in mouse RBCs pretreated with wortmannin, A6730 or M-20. *$P < 0.05$ compared with basal level, #$P < 0.05$ compared with ΔNO_2^- in the medium of cells treated only with Epo (gray bars), \$$P < 0.05$ compared with ΔNO_2^- / ΔNO_3^- in RBCs treated only with Epo (black bars); n = 6; mean ± SEM.

Fig. 9. Effect of Epo on the distribution of NO_2^-/NO_3^-. Wt RBCs were pretreated with 3 mM L-NMA for 30 min to block RBC-eNOS and incubated afterwards with 10 U/ml rHuEpo for 1 h. Results are presented as Epo-induced changes of nitrite/nitrate levels ($\Delta NO_2^- / \Delta NO_3^-$) in the absence or presence of the inhibitor. X-axis represents the basal NO_2^-/NO_3^- levels in the medium and RBCs in the absence of Epo. *A*: Blocking of RBC-eNOS caused complete inhibition of Epo-induced NO_3^- accumulation in the erythrocytes *B*: Epo was still able to trigger significant NO_2^- accumulation in the medium in the presence of L-NMA. Simultaneously, when *de novo* NO-synthesis was blocked, Epo treatment resulted in NO_2^- depletion of the RBCs. $*P < 0.05$ compared with basal level, $\#P < 0.05$ compared with ΔNO_2^- in the medium of cells treated only with Epo, $\$P < 0.05$ vs. $\Delta NO_2^- / \Delta NO_3^-$ in RBCs treated only with Epo; n = 6; mean ± SEM.

Fig. 10. Effect of L-arginine deprivation and Epo on cellular redox state. *A*: Superoxide anion production of wt RBCs was measured during 2 h of incubation period using luminescent assay in the presence or absence of 3 mM L-Arg. L-Arg deprivation resulted in pronounced increase of $*O_2^-$ generation. Administration of 1 U/ml rHuEpo in the absence eNOS substrate was accompanied by further increase in superoxide anion production. *B*: Wt erythrocytes were incubated for 2 h in the presence or absence of 3 mM L-Arg. At the beginning of the incubation RBCs were separated in several groups and treated with either saline, 1 U/ml or 100 U/ml rHuEpo. At the end of the incubation period, samples were taken for measuring of reduced and oxidized glutathione (GSH/GSSG). Half-cell reduction potential (E_{hc}) was calculated as previously described (50). eNOS substrate deprivation resulted in increased E_{hc} in comparison to the L-Arg containing control thus shifting cellular redox state to more oxidized. Epo treatment led to further oxidation of GSH. In contrary, in the presence of L-Arg low Epo levels did not affect E_{hc}. Moreover, therapeutic doses of Epo (100 U/ml) exerted

antioxidative effect by decreasing the E_{hc} and respectively altering redox potential of the RBCs to more reduced. *$P < 0.05$ compared with L-Arg containing control, #$P < 0.05$ compared with L-Arg deficient control; n = 6; mean ± SEM.

Figure 1; Mihov et al

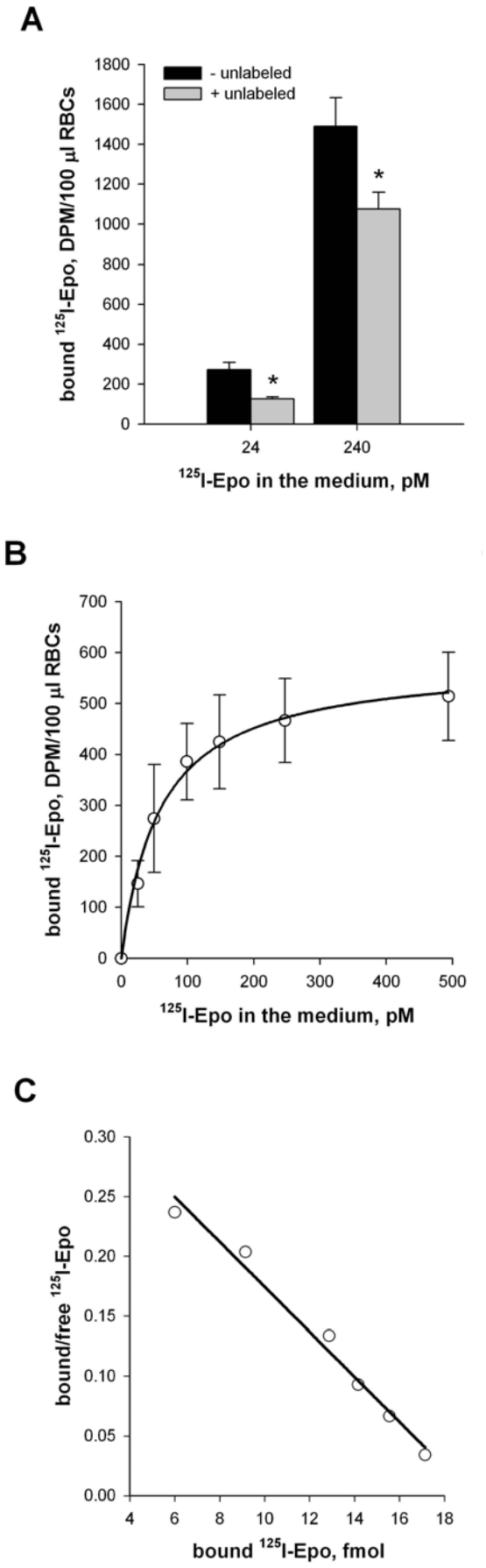


Figure 2; Mihov et al

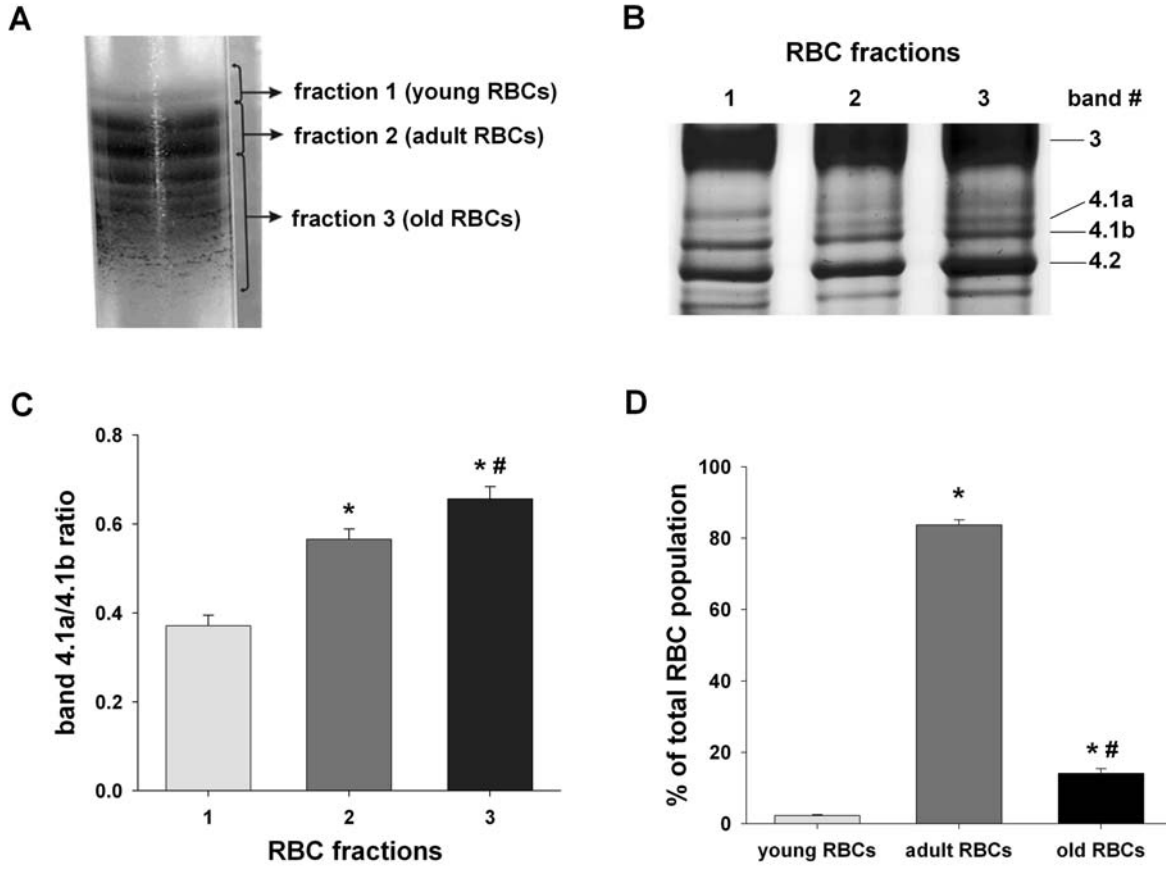


Figure 3; Mihov et al

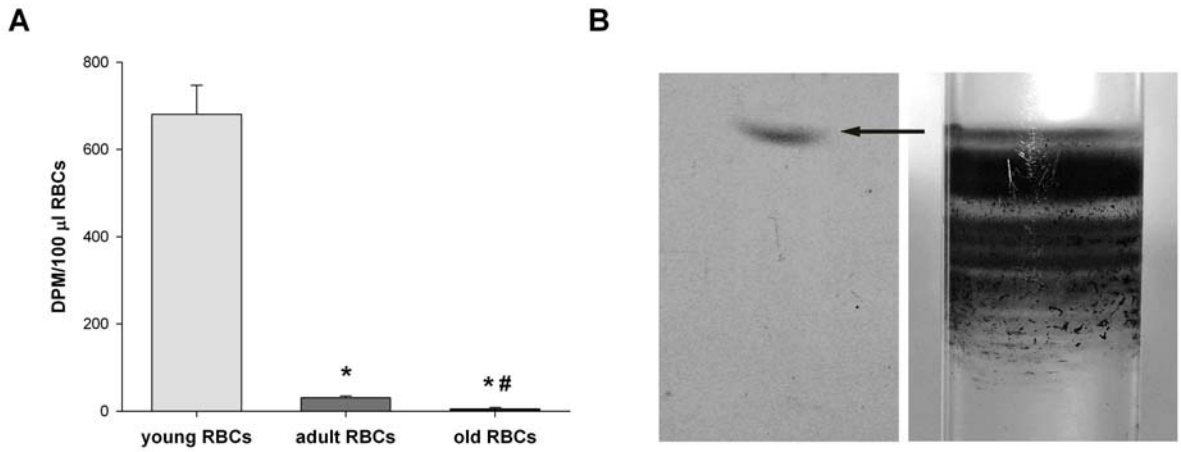


Figure 4; Mihov et al

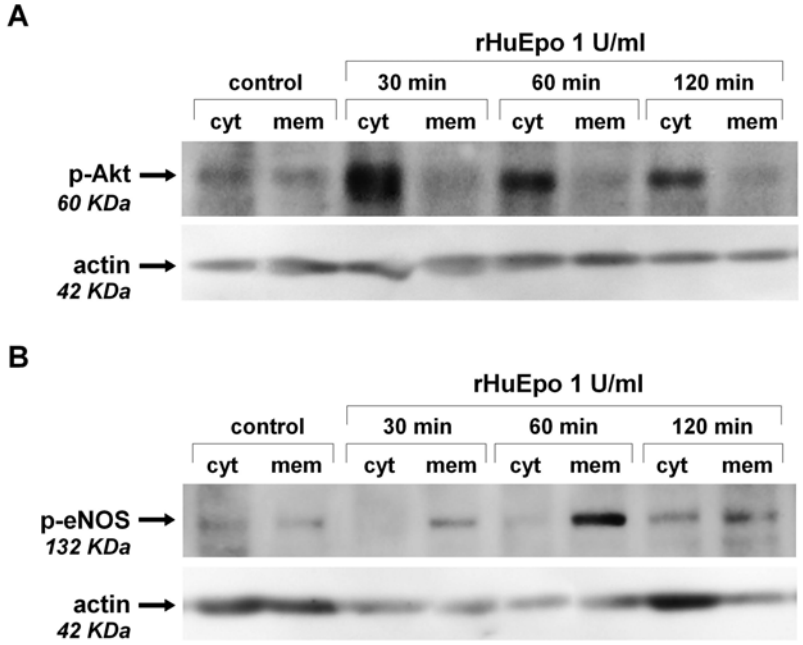


Figure 5; Mihov et al

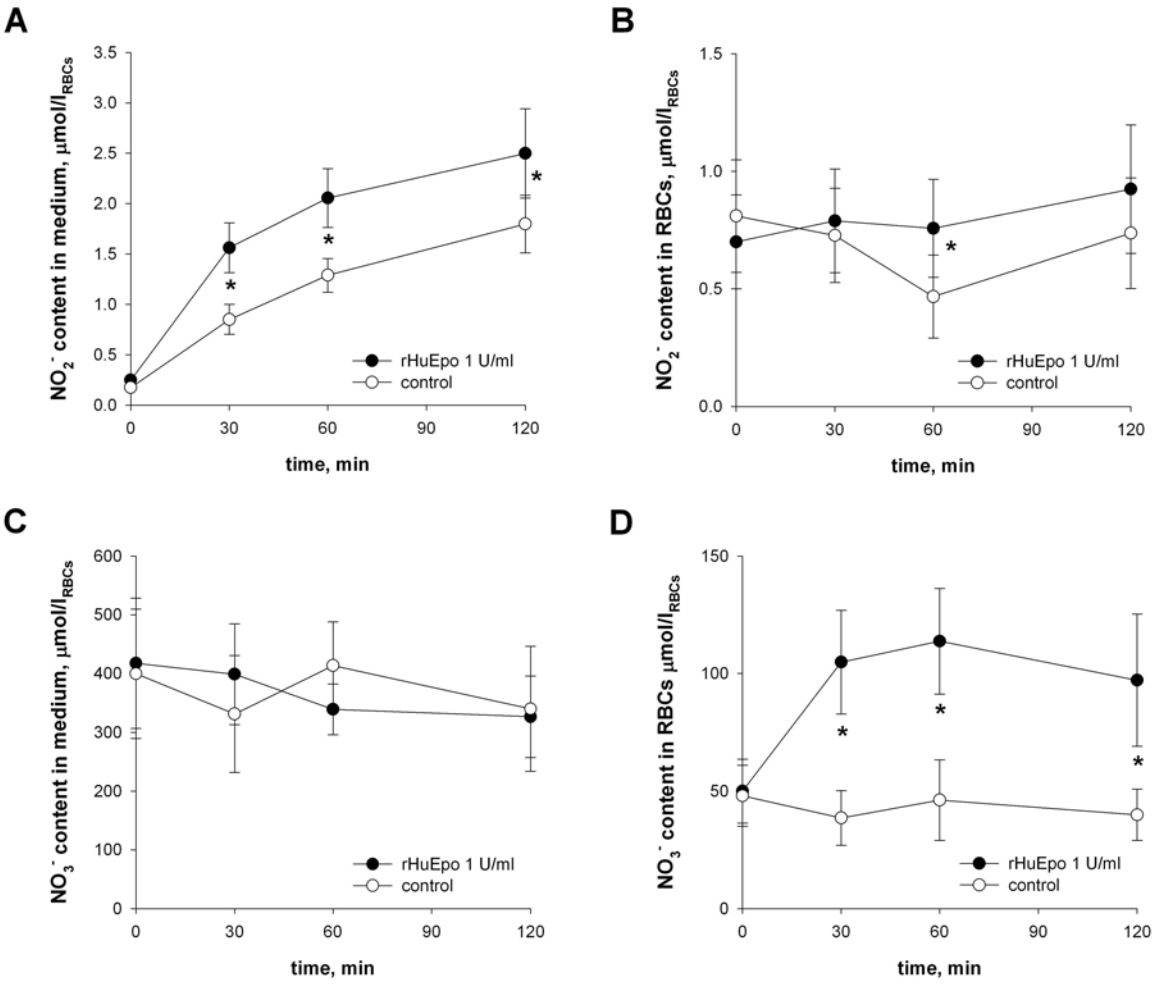


Figure 6; Mihov et al

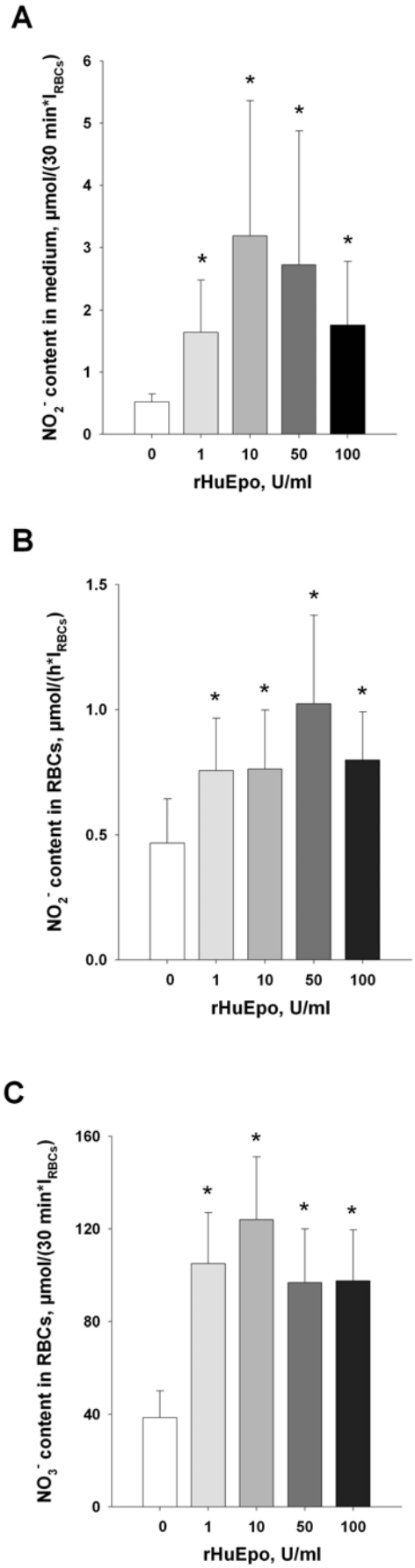


Figure 7; Mihov et al

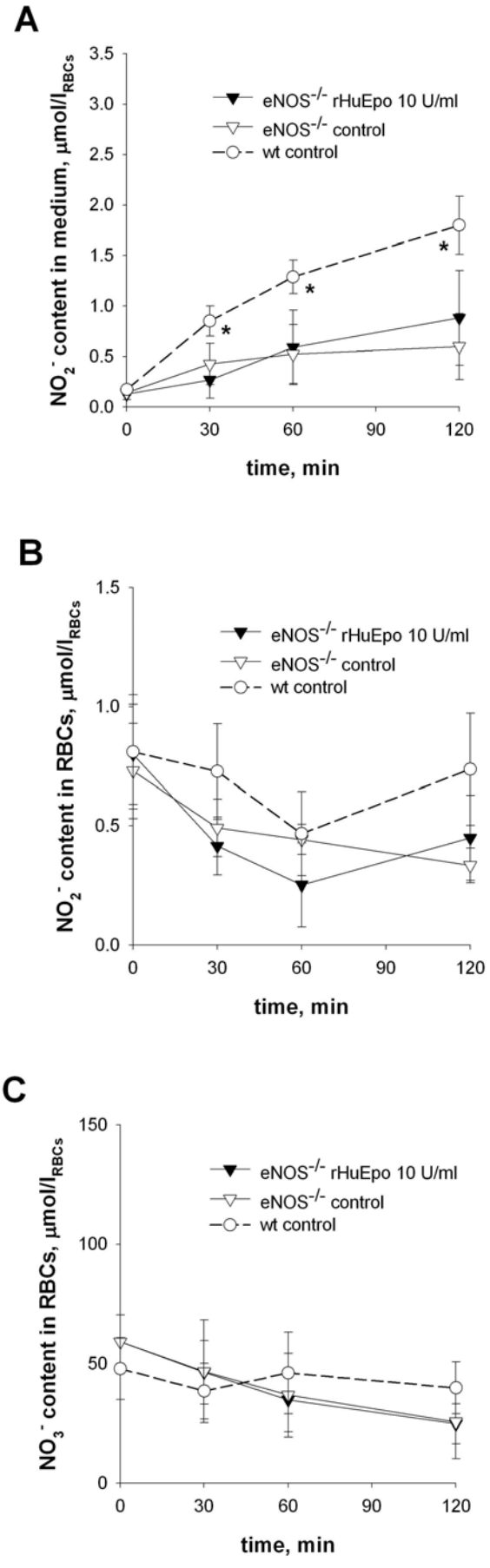


Figure 8; Mihov et al

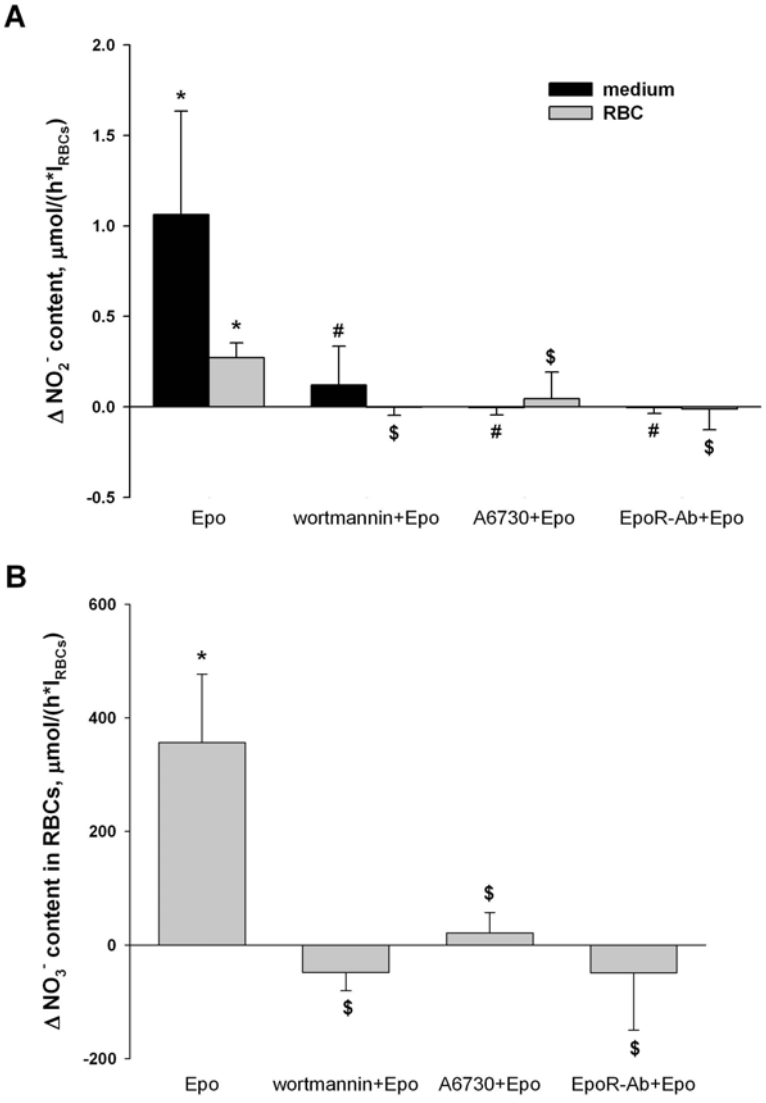


Figure 9; Mihov et al

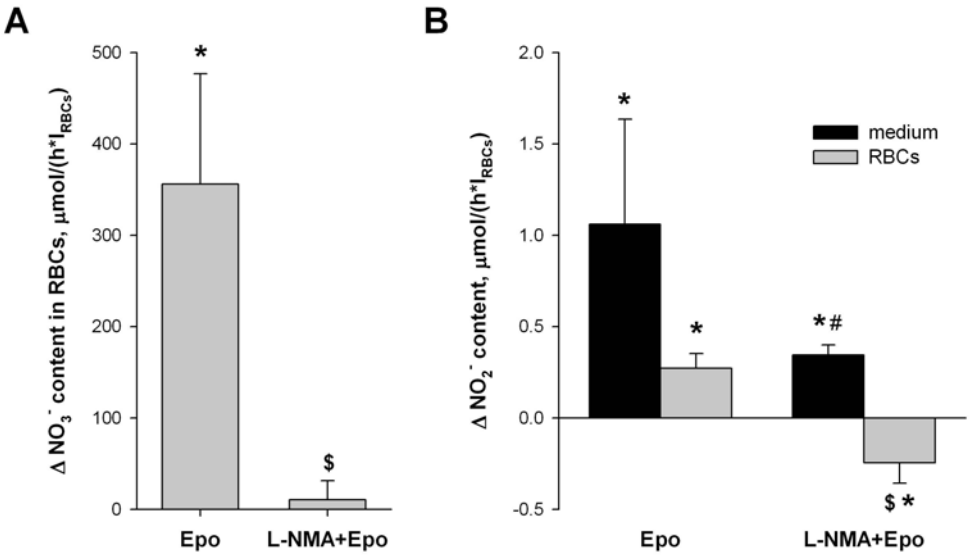
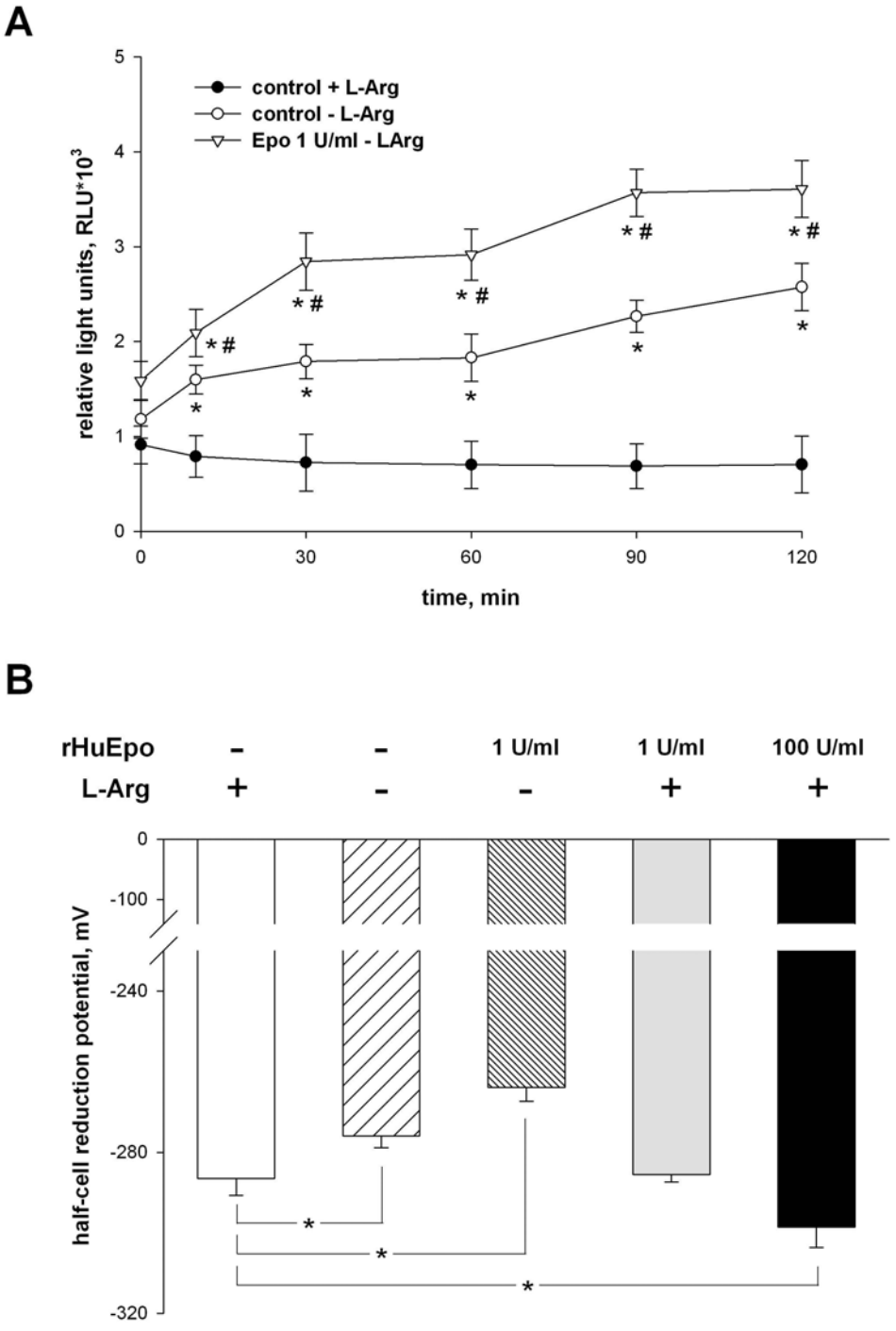


Figure 10; Mihov et al



SUPPORTING INFORMATION

Figure S1; Mihov et al

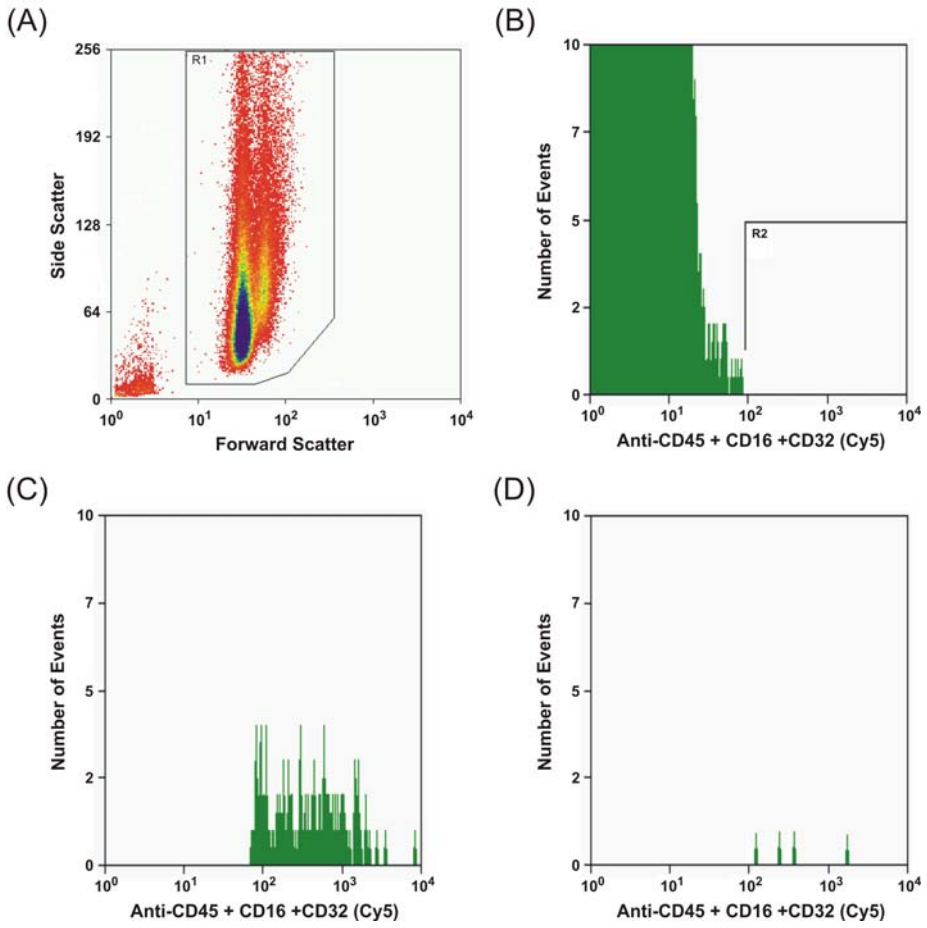


Fig S1. Gating parameters for FACS analysis of blood cell suspensions, before and after buffy coat removal. White blood cells were stained with mixture of Cy5 conjugated primary antibodies, against CD45, CD16 and CD32 (Abcam plc, Cambridge, UK). Flow cytometry was performed on a Becton Dickinson FACSCalibur and 80000 events per measurement were counted. (A) Total cell population excluding platelets and cell debris was gated (R1) based on size and granularity characteristics as measured by forward and side scatter, respectively. (B) Non treated cells from R1 were used for gating of the specific florescent signal (R2). (C) When buffy coat was present, R2 population (positive stained cells) was 0.16% ± 0.04 of the total cell number. (D) After buffy coat removal, the cells in gate R2 were 0.02% ±0.01 from the total cell population.

Figure S2; Mihov et al

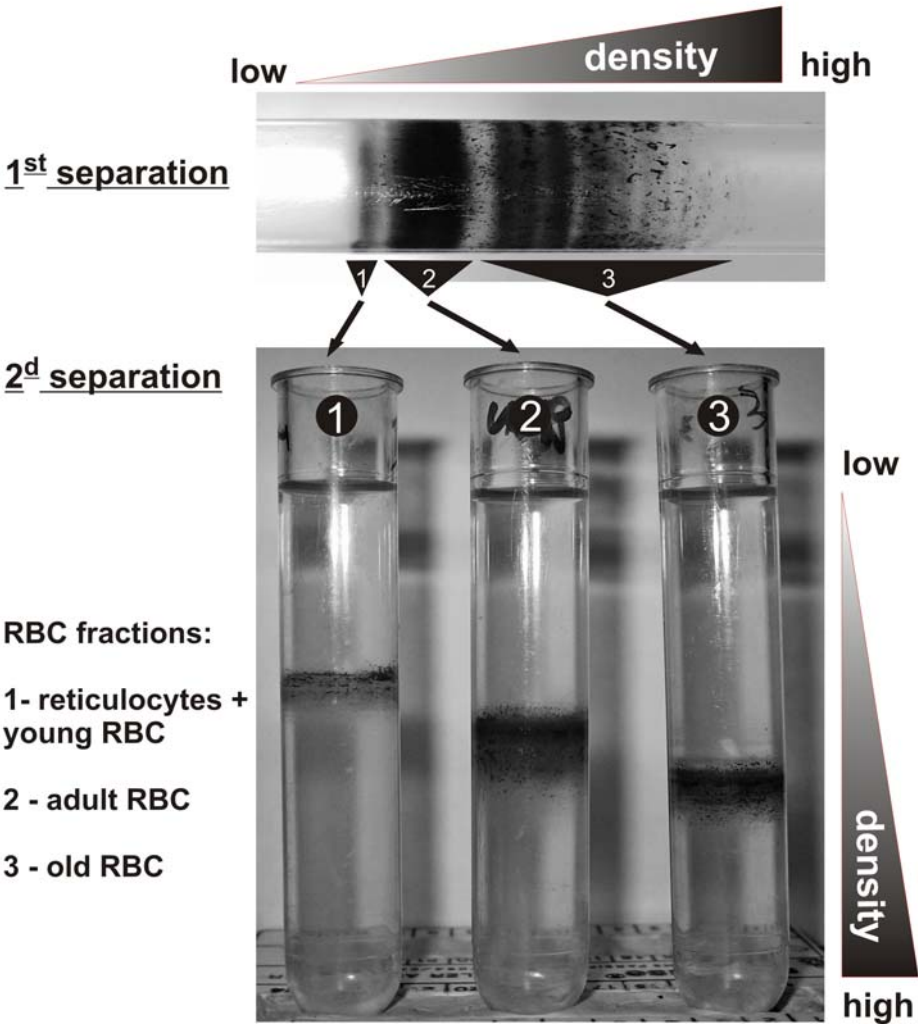


Fig S2. Repeated density separation of mouse erythrocytes. Mouse erythrocytes (0.4 ml packed RBCs) were centrifuged in 90% Percoll solution (13.5 ml) at 35000 g for 40 min (10^{o} C). Cells were carefully pipeted and divided in to three fractions: 1) reticulocytes + young RBCs; 2) adult erythrocytes; 3) old erythrocytes. Erythrocytes from the different fractions were washed twice with incubation medium and centrifuged for second time, separately, in self forming Percoll density gradient (90% Percoll, 35000g, 40 min, 10^{o} C).

8.2 Paper 2 (published manuscript)

Enhanced erythro-phagocytosis in polycythemic mice overexpressing erythropoietin

Anna Bogdanova, Deyan Mihov, Hans Lutz, Bianca Saam, Max Gassmann, and Johannes Vogel

Submitted December 19, 2006; accepted March 26, 2007. Prepublished online as *Blood* First Edition paper, March 29, 2007; DOI 10.1182/blood-2006-12-063602.

Enhanced erythro-phagocytosis in polycythemic mice overexpressing erythropoietin

Anna Bogdanova,[1] Deyan Mihov,[1] Hans Lutz,[2] Bianca Saam,[1] Max Gassmann,[1] and Johannes Vogel[1]

[1]Institute of Veterinary Physiology, Vetsuisse Faculty and Zürich Center for Integrative Human Physiology (ZIHP), University of Zürich, Switzerland; [2]Institute of Biochemistry, Zürich, Switzerland

Adaptive mechanisms to hematocrit levels of 0.9 in our erythropoietin-overexpressing mice (tg6) include increased plasma nitric oxide levels and erythrocyte flexibility. Doubled reticulocyte counts in tg6 suggest an increased erythrocyte turnover. Here we show that compared with wild-type (wt) animals, erythrocyte lifespan in tg6 is 70% lower in tg6 mice. Transgenic mice have a younger erythrocyte population as indicated by higher intercellular water and potassium content, higher flexibility, decreased density, increased surface to volume ratio, and decreased osmotic fragility. Interestingly, despite being younger, the tg6 erythrocyte population also harbors characteristics of accelerated aging such as an increased band 4.1a to 4.1b ratio, signs of oxidative stress, or decreased surface CD47 and sialic acids. In tg6, in vivo tracking of PKH26-labeled erythrocytes revealed dramatically increased erythrocyte incorporation by their liver macrophages. In vitro experiments showed that tg6 macrophages are more active than wt macrophages and that tg6 erythrocytes are more attractive for macrophages than wt ones. In conclusion, in tg6 mice erythrocyte aging is accelerated, which results, together with an increased number and activity of their macrophages, in enhanced erythrocyte clearance. Our data points toward a new mechanism down-regulating red cell mass in excessive erythrocytosis in mice. (Blood. 2007;110:762-769)

Introduction

During aging, the erythrocytes considerably change the internal ion and protein composition, and the biomechanical and biochemical properties of their cell membrane, as well as size, shape, and surface to volume ratio. In humans, these changes finally result in selective elimination of senescent erythrocytes from the circulation.[1] The clearance of erythrocytes in mice and rabbits is in part age dependent and in part random.[2] Nevertheless, the age-dependent erythrocyte clearance in these mammals is well controlled, involving oxidative damage, phosphatidylserine exposure,[3,4] desialylation,[5] and immunoglobulins.[6] The involvement of immunoglobulins in clearance of senescent erythrocytes in mice must, however, differ from that in humans, because naturally occurring antibodies in mice are exclusively of the IgM class. Under pathological conditions such as sickle cell anemia the phosphatidylserine exposure to the outer leaflet of the red cell membrane presumably plays a predominant role since in this disease the percentage of phosphatidylserine-expressing red cells is increased 2- to 10-fold.[4]

Our transgenic (tg6) mice that constitutively overexpress human Epo in an oxygen-independent manner have a 12-fold elevated Epo plasma level[7] leading to hematocrit values of up to 0.9. As to the question how tg6 mice cope with excessive erythrocytosis, we reported earlier that they show chronic vasodilatation due to excessive NO production[7] and regulate blood viscosity by elevating erythrocyte flexibility.[8] Here we were especially interested to define the process of erythrocyte turnover in tg6 animals by focusing on mechanisms of erythrocyte sequestration that prevent the polycythemic mice to further elevate their hematocrit until death. Indeed, we discovered that these animals down-regulate erythrocyte numbers by accelerated red cell aging and by highly efficient erythrocyte clearance by macrophages.

Materials and methods

Animals

The transgenic mouse line (termed tg6) overexpresses human Epo cDNA driven by the human platelet-derived growth factor B-chain promotor[7] and shows increased Epo levels in plasma and brain.[9] Breeding was performed by mating hemizygous males to wild-type C57Bl/6 females. Half of the offspring was hemizygous for the transgene while the other half was wild type (wt) and served as controls. Animals used for assessment of erythrocyte properties were killed with CO_2. Then the blood was collected through cardiac puncture into heparinized syringes. All experiments were performed in accordance with the Swiss animal protection laws (Kantonales Veterinäramt Zürich) and institutional guidelines.

Measurements of the unidirectional K^+ influx and cellular ion and water content

The unidirectional K^+ influx measurements in mouse erythrocytes are described in detail elsewhere.[10] Briefly, blood from 2 to 3 animals was pooled, and erythrocytes were isolated and suspended in the incubation medium (125 NaCl, 25 $NaHCO_3$, 5 KCl, 1 $CaCl_2$, 0.15 $MgCl_2$, 10 Tris-MOPS, 10 glucose, 10 sucrose, all in mM, pH 7.4). Incubation was performed in Eschweiler tonometers with a gas phase consisting of 5% CO_2, 20% O_2, and 75% N_2. The cells were preincubated for 20 minutes with or without inhibitors of the Na/K ATPase (1 mM ouabain) or Na-K-2Cl cotransporter (NKCC, 100 μmol bumetanide). Thereafter, ^{86}Rb was added as a radioactive tracer for K^+ and aliquots of the suspension were taken 20, 40, and 60 minutes after addition of the tracer. Flux was terminated by

Submitted December 19, 2006; accepted March 26, 2007. Prepublished online as *Blood* First Edition paper, March 29, 2007; DOI 10.1182/blood-2006-12-063602.

The publication costs of this article were defrayed in part by page charge payment. Therefore, and solely to indicate this fact, this article is hereby marked "advertisement" in accordance with 18 USC section 1734.

immediate dilution of the ^{86}Rb with excessive amounts of ice-cold washing solution containing 100 mM $Mg(NO_3)$ and 10 mM imidazol titrated with HNO_3 to a pH of 7.4 when on ice. Cells were washed 3 times to remove not internalized radioactivity and then deproteinized with 0.2 mL of 5% trichloracetic acid. The amount of ^{86}Rb in the medium and deproteinized cell pellet extracts were measured in water phase (Cherenkov effect) using a Tri-Carb 1600TR liquid scintillation counter (Packard, Palo Alto, CA).

For the measurements of ion/water content of erythrocytes, blood was immediately centrifuged for 5 minutes at 6000*g* and 4°C in predried preweighed Eppendorf tubes. Plasma and buffy coat were discarded and erythrocytes were washed 3 times with an ice-cooled buffer. Packed cell pellets were weighed and dried at 80°C for 72 hours. After reweighing, the dried pellets were burned in ultra-pure concentrated HNO_3 and cellular Na^+ and K^+ content was determined using a flame photometer.

Osmotic gradient ektacytometry

Erythrocyte deformability was measured using laser diffraction technique with an ektacytometer (Technikon Products, Bayer, Germany). Erythrocytes were suspended in a viscous solution containing Dextran T70 (GE Healthcare, Uppsala, Sweden) and osmoscans were recorded as described elsewhere.[11]

Osmotic fragility

Blood (10 μL) was dissolved in 3 mL distilled water containing either 0.9%, 0.75%, 0.65%, 0.55%, 0.5%, 0.45%, 0.4%, 0.35%, 0.3%, 0.2%, or 0% NaCl each adjusted to a pH of 7.4 using concentrated HCl. Ten minutes later, the tubes were centrifuged for 5 minutes at 2000*g*. Then 2 mL of the supernatant was added to 1 mL hemoglobin transformation solution (Dr Lange AG, Hegnau, Switzerland), mixed, and measured photometrically at 546 nm. The extinction was then plotted on a percentage basis of the hemolysis in distilled water (set to 100%) against the NaCl concentration in the test solution. The NaCl concentration at 50% hemolysis was determined by regression analysis.

Erythrocyte life span

Erythrocytes were labeled ex vivo with Biotin-x-N-hydroxysuccimide ester (BxNHS; Calbiochem, Dietikon, Switzerland) according to Hoffmann-Fezer et al.[12] In brief, anesthesia was induced with a gas mixture containing 4% halothane, 70% N_2O, remainder O_2 and maintained by reducing the inspired halothane concentration to 1% to 1.5%. Body temperature was maintained at 37°C using a temperature-controlled heating pad. A catheter was inserted into the left femoral vein and 300 (wt) to 600 (tg6) μL blood was withdrawn into a syringe containing about 25 units of heparin. The blood loss was substituted with 200 (wt) to 400 (tg6) μL saline. The blood was then incubated with 1 mg BxNHS dissolved in 20 μL dimethylformamide and 120 μL phosphate-buffered saline (PBS) containing 0.1% glucose (PBS-G) at 37°C for 30 minutes with gentle shaking. Then the erythrocytes were washed twice with PBS-G, resuspended in PBS-G, and reinfused via the catheter in the femoral vein. After removal of the catheter and wound closure the mice were housed under standard conditions. Then every 3 to 4 days, approximately 2 μL blood was collected from each animal and incubated for 10 minutes with FITC-labeled avidin (Calbiochem) before determining the percentage of labeled erythrocytes with a fluorescence-activated cell sorting (FACS) analyzer (FACSCalibur; Becton Dickinson, Oxford, United Kingdom). Sampling was stopped when less than 0.5% labeled cells was found. The percentage of labeled erythrocytes was plotted against the day of blood collection and erythrocyte half life was calculated for each individual animal using exponential regression analysis.

In vivo erythrocyte tracking

Blood was collected as described in "Erythrocyte life span." After washing the erythrocytes twice with PBS-G containing 0.2 mM phenylmethylsulfonyl fluoride (PMSF), the packed cells were diluted in "diluent C" of the PKH26-labeling Kit (Sigma, Buchs, Switzerland) and mixed with the same volume of "diluent C" containing 4 μM of the dye according to the supplier's instructions. The erythrocytes were then incubated at 37°C for 20 minutes under constant agitation. Four days after reinfusion of the labeled erythrocytes, the presence of labeled erythrocytes was confirmed by analyzing a blood smear and the animals were transcardially perfused with PBS containing 25 IU heparin/mL at 115 mmHg for at least 10 minutes to wash away all erythrocytes from the vasculature. Finally, fixation was achieved by perfusing with 3% paraformaldehyde (PFA) in PBS. Thereafter, liver and spleen were excised, stored for 3 days in 3% PFA followed by 4 days storage in 20% sucrose in PBS, and frozen for further analysis. In one mouse, in addition to these 2 organs also brain, kidney, heart, adrenal gland, muscle, and gut with Peyer patches were harvested and analyzed. Except for liver and spleen, no PKH26 fluorescence was detected in any other organ, confirming the complete removal of free erythrocytes from the animals' organs (not shown).

Subsequently, liver and spleen were sectioned into 10-μm slices using a cryomicrotome and stained with a FITC-labeled rat antibody directed against the F4/80 antigen (dilution 1:100; THP, Vienna, Austria) that is known to be present on the major subpopulation of resident tissue macrophages.[13,14] Sections were analyzed using an incident fluorescence microscope (Axiovert 200M, Plan-Neofluar objective lens 20×/0.50; Zeiss, Deisenhofen, Germany) and arbitrary areas of liver and spleen were documented using an Axiocam HRm CCD camera and Zeiss Axiovision software version 4.5 (Zeiss). The resulting images were used to assess the total area of PKH26 fluorescence per tissue area. The area occupied by empty vessels was excluded using an image analyzing system (MCID Analysis 7.0, St. Catharines, ON) that allows to discriminate the vessels from the slightly autofluorescent tissue by their nearly black appearance. Tissue area could then be calculated from the difference of the total image area and the vessel area. Incorporated erythrocytes appeared as white dots within the tissue (Figure 3B).

Macrophage assay

These experiments were performed according to Bratosin et al[15,16] with slight modifications. In brief, peritoneal macrophages were isolated by washing the peritoneal cavity with 10 mL Hanks balanced salt solution (HBSS) followed by centrifugation of the washing solution. The cells were then resuspended in 1 mL DMEM containing 20% FCS and incubated for 4 hours at 37°C in humidified air containing 5% CO_2. Nonadherent macrophages were removed by 2 washings with DMEM. Then PKH26-labeled erythrocytes were added to the macrophages (macrophage-erythrocyte ratio = 1:10, approximately 2×10^6 cells/mL). After incubation for an additional 2 hours, unbound erythrocytes were removed by washing with DMEM. The remaining unincorporated erythrocytes were lysed using a hypotone buffer (140 mM NH_4Cl, 17 mM Tris, pH 7.2) and the macrophages were fixed by flushing the Petri dishes with 3% paraformaldehyde in PBS and treated with 70% ethanol in order to elute the PKH26 from ghosts still adherent to the Petri dishes or macrophages. Arbitrary areas of the macrophage culture were then photographed at dark field and at appropriate fluorescence excitation light for PKH26. Using the image analyzing system mentioned in "Materials and methods, In vivo erythrocyte tracking," the percentage of labeled macrophages and the total fluorescent area within each single macrophage containing labeled erythrocytes were determined.

Assessment of properties typical for senescent erythrocytes

Direct Coombs test. Erythrocytes of tg6 and wt mice were washed 3 times in 20 volumes of PBS-G. Thereafter, packed cells (centrifuged at 350*g*) were mixed with 5 volumes of pure, 1:1, 1:3, 1:7, 1:15 diluted antimouse polyvalent immunoglobulin (anti-IgG, anti-IgM whole molecule; Sigma) and PBS-G as negative control. Ten minutes later, the agglutination was recorded semiquantitatively.

Band 4.1a to 4.1b ratio. After washing erythrocytes 3 times in PBS-G containing 30 μg/mL PMSF, they were hemolysed in 30 volumes of lysis buffer (5 Na_2HPO_4, 1 EDTA, 0.4 di-isopropyl fluorophosphate, all in mM, pH 7.4). Then the erythrocyte ghosts were washed several times in lysis buffer until the pellet appeared completely pale after centrifugation (Sorvall RC-5B rotor (Thermo Electron Corp., Franklin, MA): SS34 at 47800*g*, 20°C, 10 minutes). Packed membranes were then mixed with one volume of a solution containing 1% sodium dodecyl sulfate (SDS) and 5 mM N-ethylmalaeimide (NEM) and frozen at −70°C. After thawing and

determination of the protein content, the samples were mixed with electrophoresis buffer containing 40 mM DTT and heated to 100°C for 3 minutes. Then an excess of NEM was added and the proteins were separated on 8% SDS–polyacrylamide gels and visualized using silver staining as described elsewhere.[17] The ratio of band 4.1a and 4.1b was quantified using the image analyzing system mentioned above.

Nonprotein thiols. The amount of cellular glutathione (GSH) and oxidized glutathione (GSSG) was assayed in erythrocytes as described elsewhere.[10] Briefly, blood and plasma samples were collected from wt and tg6 mice and mixed 1:1 with deproteinizing solution containing 1.67 g glacial metaphosphoric acid, 0.2 g Na_2EDTA, and 30 g NaCl in 100 mL ddH_2O. After centrifugation, GSH and GSSG (upon reduction to GSH in the presence of glutathione reductase and NADH) concentration was determined in supernatants using 5,5′-dithiobis(2-nitrobenzoic acid) (Ellman reagent). Optical density of the colored complex was measured photometrically at 412 nm.

CD47. The amount of CD47 on the surface of erythrocytes was determined using a FITC-labeled monoclonal antibody directed against mouse CD47 (BD Biochemistry, Allschwil, Switzerland). After 3 washes with PBS-G and 1-hour incubation at room temperature with the antibody diluted 1:50, 1:100 (recommendation of the supplier), or 1:200, fluorescence intensity was quantified by FACS.

Surface sialic acids. The amount of sialic acid residues on the erythrocyte surface was determined as described by Aminoff.[18] Briefly, mouse erythrocytes were washed twice in 0.9% NaCl and finally in a solution containing 10 mM $CaCl_2$ and 0.9% NaCl titrated with $NaHCO_3$ to a pH of 7.0 at 37°C. Final hematocrit of the erythrocyte suspension was adjusted to 17% to 25% using the same solution. Aliquots of the suspension (200 μL) were incubated with neuraminidase (final activity of 0.2 U/mL) for 1 hour at 37°C. After centrifugation (5 minutes at 2000*g*), the supernatants were collected and deproteinized by boiling for 2 to 3 minutes. After acidification to a pH of 4.0 with 1 drop of glacial acetic acid, 100 μL of the deproteinized supernatant was mixed with 50 μL periodic acid (25 mM of periodic acid in 0.125 N H_2SO_4) and incubated for additional 30 minutes at 37°C. Thereafter the excess of periodate was neutralized by adding 40 μL sodium arsenide (2% in 0.5 N HCl) and the mixture incubated with 400 μL 2-thiobarbituric acid for 7.5 minutes at 100°C in a water bath. The colored complex was then extracted with 1 mL acidic butan-1-ol and absorbance was measured photometrically at 549 nm. A calibration curve was made using 1-40 μg *N*-acetylneuraminic acid dissolved in distilled water.

In addition, a qualitative test for assessment of surface sialic acids was performed[19] by mixing one drop of PBS-G washed erythrocyte suspension adjusted to a hematocrit of 0.2 with 2 drops of PBS containing 1% hexadimetrin bromide (Polybrene; Sigma) at room temperature. Ten minutes later, the suspension was transferred to Neubauer chambers and observed for red cell aggregation in an Axioskop 2 microscope with a Plan-Neofluar 20×/0.50 objective lens. Images were captured using an AxioCam CCD color camera and processed using AxioVision 4.2 and converted to grayscale.

Statistical analysis

Data were compared among the experimental groups using ANOVA and a 2-tailed Student *t* test for unpaired samples with Welch correction or a Fischer test (GraphPad Instat. V3.05; GraphPad Software, San Diego, CA). The level of statistical significance was set at $P < .05$.

Results

Ion and water content

The plasma concentrations of the major cations did not differ between tg6 and wt mice (not shown). In contrast, both K^+ and water content of tg6 erythrocytes exceeded that of the wt, whereas Na^+ concentration was similar (Figure 1A). Figure 1B shows that the enhancement of the active (ouabain-sensitive, ATPase-mediated) K^+ influx in transgenic erythrocytes is coupled to a partial suppression of the passive (bumeteanude-sensitive, NKCC-mediated) K^+ flux. The transmembrane electrochemical gradient for potassium directed NKCC-mediated net K^+ flux outwardly. Thus, its suppression along with activation of ouabain-sensitive K^+ uptake explains the increased intracellular K^+ content of transgenic erythrocytes that is typical for young erythrocytes.

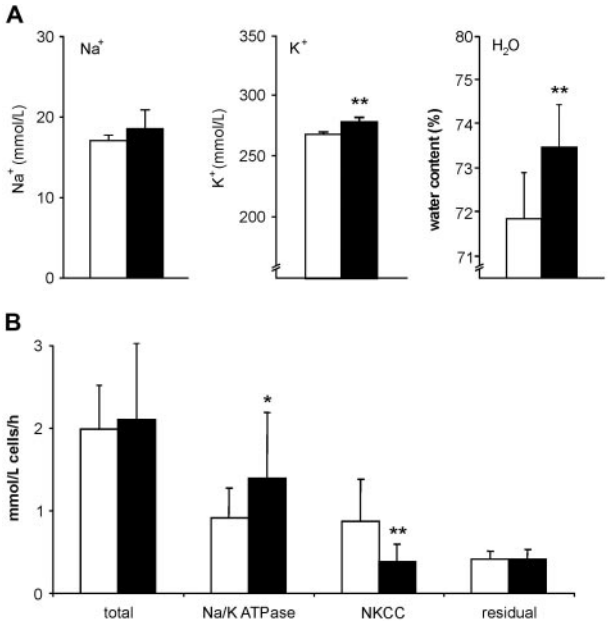


Figure 1. Concentration of the major intracellular cations, water content, and potassium fluxes of wt (open bars) and tg6 (black bars) erythrocytes. (A) The intracellular sodium concentration was unaffected, whereas the potassium concentration was significantly elevated in tg6 erythrocytes. Accordingly, the water content of tg6 erythrocytes was higher compared with wt ones. (B) The increased intracellular potassium concentration was due to an increased ouabain-sensitive (active, Na/K-ATPase) transmembral potassium flux coupled with a suppression of the bumetanide-sensitive (passive, NKCC) flux. Means ± SD; $*P < .05$; $**P < .01$.

Osmotic gradient ektacytometry

Apart from the osmolarity of the suspension medium, the deformability of the erythrocytes is determined by their membrane elasticity, their surface to volume ratio, and their internal viscosity, the latter being a function of the mean corpuscular hemoglobin concentration that is inversely proportional to the density of the erythrocytes. Osmoscans were performed with erythrocytes from 3 to 4 individual wt or tg6 mice. The osmoscans (not shown) were highly reproducible and revealed that, compared with the wt control, tg6 erythrocytes have an increased surface to volume ratio, a reduced cell density, and an increased flexibility. Enhanced flexibility confirms our previous results[8] and fits, together with the decreased cell density, to the alterations in ion and water content shown in Figure 1. Once again, these observations point toward the presence of a younger erythrocyte population.

Osmotic fragility

Considering that the erythrocyte population in tg6 mice is more juvenile, we tested their resistance to osmotic stress. While 50% of the wt erythrocytes lysed at a NaCl concentration of 0.54% ± 0.04%, tg6 erythrocytes were more stable, lysing at 0.43% ± 0.04% NaCl ($n = 7$, $P < .05$). Most likely the lower osmotic fragility of the tg6 erythrocytes is due to their higher surface to volume ratio.

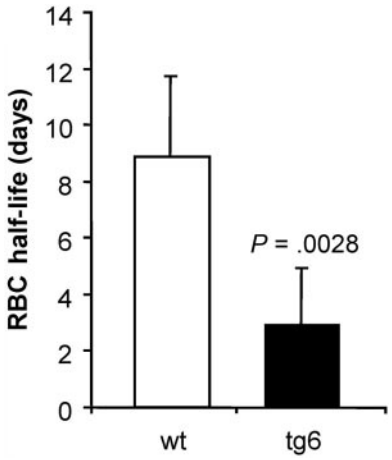


Figure 2. Decay of biotin-x-N-hydroxysuccimide ester–labeled erythrocytes. The percentage of labeled cells (day 0, set to 100%) was measured every 3 to 4 days until less than 0.5% labeled cells was found and the data were plotted against the day of sampling. For each individual mouse, an exponential regression equation was determined that was used to calculate the erythrocytes' half live as shown in the figure. Life span of tg6 erythrocytes is reduced to almost a third compared with wt erythrocytes. Means ± SD; n = 5-6.

Red cell life span

The life span of wt erythrocytes was in accordance with the values reported in the literature[20] namely about 60 days. Interestingly, in tg6 mice the life span of the erythrocytes was reduced to almost a third compared with wt (Figure 2). Obviously, there are mechanism(s) that allow efficient removal of transgenic erythrocytes, thereby counteracting the fatal effects of excessive erythrocytosis in our tg6 mice. Tg6 red cell survival in vivo was also measured in wt mice and wt red cell survival in tg6 mice. In both experiments, the percentage of labeled cells dropped below 0.5% within 8 to 12 days (data not shown), confirming the abnormal red cell life span in tg6 mice.

In vivo red cell tracking

Four days after reinfusion, PKH26-labeled autologous erythrocytes were still present in the circulation as confirmed by blood smears. PKH26 fluorescence was detected in spleen and liver only, but in none of the other organs tested (not shown). In spleen and liver, PKH26 colocalized with F4/80 staining, indicating that the labeled erythrocytes were indeed incorporated by tissue macrophages.[14] Moreover, Figure 3A shows major differences in the distribution of the incorporated erythrocytes between spleen and liver in wt and tg6 mice. The wt spleen contained many F4/80-positive cells, mainly in the red pulp. Especially at the marginal zone between white and red pulp, macrophages were highly positive for PKH26. Interestingly, F4/80 as well as colocalized PKH26 staining was reduced in the tg6 spleen. Very low F4/80 or PKH26 staining was found in the wt liver, whereas the tg6 liver contained an increased number of F4/80-positive cells that had incorporated PKH26-labeled erythrocytes. The PKH26-positive area per tissue area in tg6 spleen, about 22 times smaller in and tg6 liver, was 150 times larger compared with wt organs (Figure 3C). Most likely, tg6 splenic macrophages were superseded by the dramatically increased number of erythropoietic precursor cells populating the transgenic spleen.[8] Considering the weight of the liver (tg6: 1.503 ± 0.18 g; wt: 0.997 ± 0.05 g; $P < .05$) and spleen (tg6: 407 ± 43 mg; wt: 87 ± 8.5 mg[8]) together with the PKH26-positive area per tissue area, tg6 mice have a nearly 8 times increased macrophage mass than do incorporate erythrocytes.

Macrophage assay

Next we exposed macrophages isolated from wt and tg6 mice to either wt or transgenic erythrocytes. Figure 4 shows that tg6 macrophages coincubated with tg6 erythrocytes had the highest phagocytic activity followed by wt macrophages coincubated with tg6 erythrocytes. In contrast, the overall phagocytic activity of wt macrophages was lower than that of tg6 macrophages. Of note, tg6 erythrocytes showed a tendency to be more attractive also for the wt macrophages. Moreover, tg6 macrophages coincubated with tg6 erythrocytes incorporated significantly more erythrocytes per single macrophage than all other combinations of macrophages and erythrocytes (Figure 4). Taken together, tg6 macrophages have an increased erythro-phagocytic activity compared with wt macrophages and tg6 erythrocytes are more attractive for both tg6 and wt macrophages.

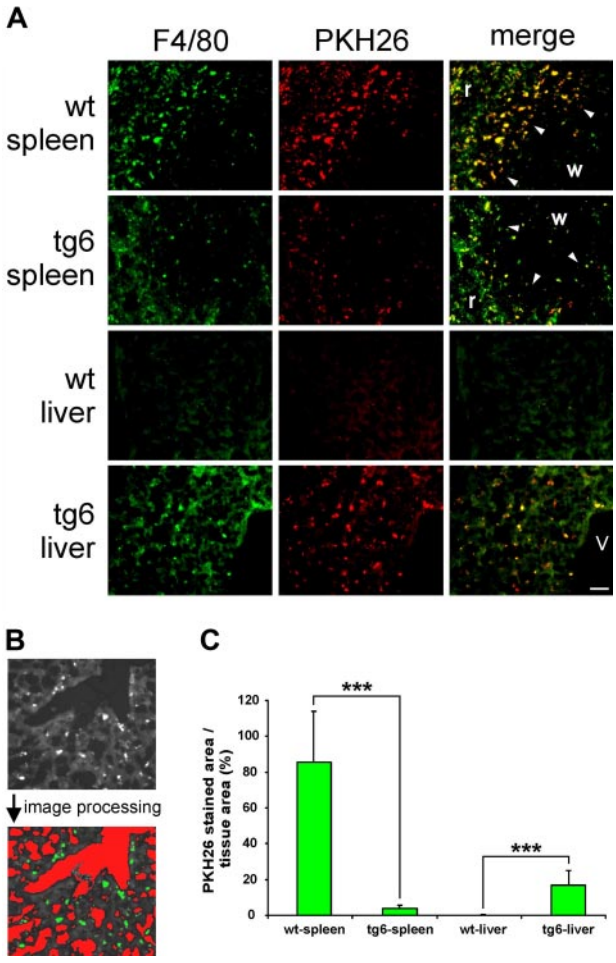


Figure 3. In vivo tracking of erythrocytes. (A) Macrophages were visualized by F4/80 staining and incorporated erythrocytes, by prior PKH26 labeling. Compared with the wt organ, F4/80 and PKH26 staining is decreased in the tg6 spleen. The most intense phagocytosis of erythrocytes was found in the marginal zone (arrowheads) between white (w) and red (r) pulp. In contrast, F4/80 as well as PKH26 staining was dramatically increased in the tg6 liver (same magnification of all images; bar represents 50 μm; v indicates central vein). (B) Illustration of the quantification procedure of the PKH26 fluorescent area using image analysis. Tissue area was defined as the total image area minus the cross-sectional area of all vessels (higher optical density compared with the tissue, marked red after image processing). The PKH26-stained area is evident from a much lower optical density compared with the tissue (marked green after image processing). (C) Compared with wt, the PKH26-positive area per tissue area was about 22 times smaller in the transgenic spleen but approximately 150 times larger in the liver of tg6 mice. Means (± SD) of 10 analyzed images of liver and spleen each of 4 animals of each line; ***$P < .001$.

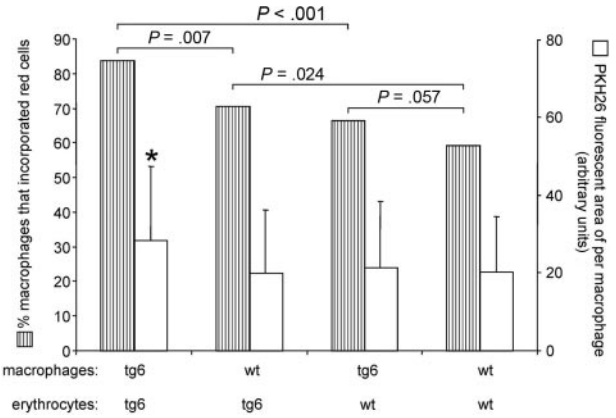


Figure 4. Assessment of macrophage activity. Hatched bars represent the percentage of macrophages that incorporated at least one red cell. Tg6 macrophages incubated together with tg6 erythrocytes showed the highest phagocytotic activity followed by wt macrophages incubated with tg6 erythrocytes. Wt macrophages were less active compared with tg6 macrophages when coincubated with tg6 or wt erythrocytes, respectively. Tg6 erythrocytes were significantly more attractive than wt erythrocytes for both tg6 and wt macrophages. Moreover, the PKH26 fluorescent area of per single macrophage (open bars) was significantly larger in tg6 macrophages coincubated with tg6 erythrocytes compared with all other combinations of macrophages and erythrocytes. *P* values for hatched bars were calculated using a Fischer test, and for open bars using a 2-tailed Student *t* test for unpaired samples. Means ± SD of 4 independent experiments each; $^*P < .05$.

Assessment of properties typical for senescent erythrocytes

In the last set of experiments, we aimed to determine the mechanism(s) leading to enhanced phagocytosis of transgenic erythrocytes.

Testing for autoantibodies against erythrocytes. To exclude that tg6 develop autoantibodies against their erythrocytes explaining their reduced life span, we performed a series of direct Coombs assays. The semiquantitative assessment of agglutination of erythrocytes suspended in different dilutions of antimouse polyvalent immunoglobulin revealed slightly higher antibody binding in wt compared with tg6 erythrocytes (not shown). Thus, we have no evidence that tg6 animals develop autoantibodies against their own erythrocytes.

Band 4.1a to 4.1b ratio. Erythrocyte ghost proteins showed an increased band 4.1a to 4.1b ratio in the whole tg6 erythrocyte population (Figure 5). While the value for control mice was in accordance to values determined earlier by others,[22] the ratio in tg6 mice was unexpectedly higher, suggesting that the tg6 erythrocyte population contains more aged than young cells. Indeed, density separation of tg6 erythrocytes revealed a different density distribution compared with wt controls with, most importantly, much lighter cells in tg6 mice (not shown).

Nonprotein thiols. As tg6 mice developed excessive erythrocytosis that nearly doubled their arterial oxygen content,[23] we speculated that tg6 erythrocytes suffer from oxidative damage. Thus, we measured the nonprotein thiols in tg6 and wt erythrocytes. Transgenic erythrocytes contained significantly less GSH compared with wt erythrocytes (2.04 ± 0.2 vs 2.55 ± 0.14 μmol/mL erythrocytes, $n = 9$, $P < .05$). Accordingly, GSSG levels of tg6 erythrocytes were slightly higher (wt: 0.031 ± 0.013 vs tg6: 0.073 ± 0.02 μmol/mL erythrocytes, $n = 8$), although the latter observation did not reach statistical significance. These findings indicate an increased in vivo oxidative stress of tg6 erythrocytes.

CD47. Elevated phagocytosis of transgenic erythrocytes might be due to reduced expression of CD47 that is known to inhibit erythro-phagocytosis by binding the inhibitory receptor signal regulatory protein alpha (SIRPα) present on macrophages.[14,24] Table 1 shows the histogram statistics of the FACS analysis of labeled (monoclonal antimouse CD47 antibody, dilution: 1:100) erythrocytes obtained from wt and tg6 mice (5-6 individual animals each). At this dilution and both other dilutions tested (1:50 and 1:200, not shown), mean, geometrical mean, median, and fluorescence intensity channel with the highest counts (peak channel) were highly significantly shifted toward lower fluorescence intensities in histograms obtained with tg6 erythrocytes. This clearly indicates a reduced expression of CD47 on transgenic erythrocytes.

In addition, the histograms of tg6 erythrocytes appeared to be wider compared with those of wt erythrocytes, suggesting a higher variance of the fluorescence intensities. This can be quantified by the standard deviation, the coefficient of variation, and the height of the peak. For transgenic erythrocytes, the peak height was significantly lower at all dilutions of the antibody, the coefficient of variation was significantly higher only at the highest dilution, and the standard deviation, at none of the dilutions.

Surface sialic acids. While aging, erythrocytes lose sialic acids[25-28] among other surface carbohydrates. As a reduction of sialic acids on the erythrocyte's surface is known to be associated with elevated erythro-phagocytosis,[16] we quantified sialic acid on wt and tg6 erythrocytes. Sialic acids at the cell's surface were 22% lower in tg6 mice compared with wt (Figure 6A). Furthermore, this observation was qualitatively confirmed by the lower tendency of tg6 erythrocytes to agglutinate in PBS containing 1% polybrene (Figure 6B).

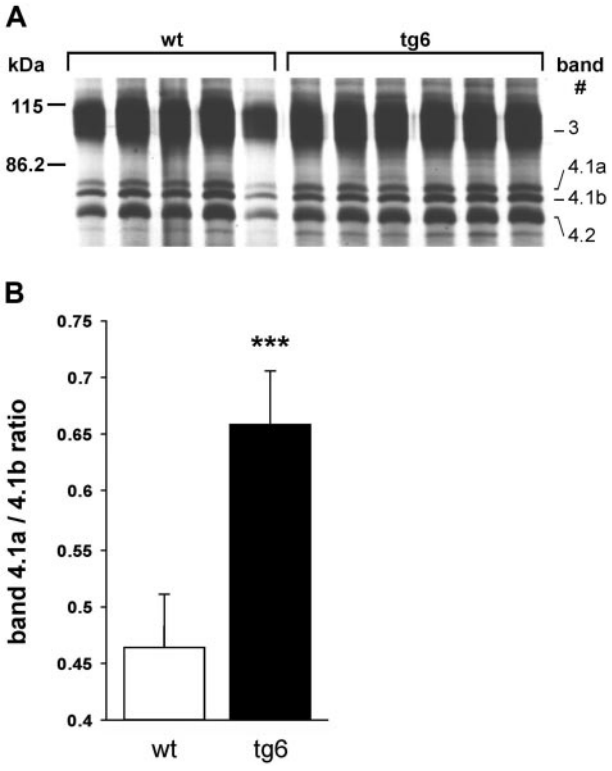


Figure 5. Band 4.1a to 4.1b ratio. (A) Silver-stained SDS–polyacrylamide gel electrophoresis (PAGE) of erythrocyte membrane proteins. Samples were isolated form 5 wt and 6 tg6 individuals and the part of the gel containing bands 3 through 4.2 labeled according to Steck[21] is shown. (B) Quantification of the band 4.1a to 4.1b ratio by densitometry revealed an increased ratio in tg6 red cell membranes, which is typical for senescent erythrocytes. Means (± SD); $n = 5$-6; $^{***}P < .001$.

Table 1. Histogram statistics of FACS analysis of erythrocytes incubated with an FITC-labeled anti-CD47 antibody

	Mean	Geometrical mean	SD	CV	Median	Peak	Peak channel
Wt							
Mean	75.4	68.8	42.8	56.6	70.4	653.0	68.8
SD	2.8	1.7	6.2	6.0	1.7	9.9	3.3
tg6							
Mean	65.8	59.1	41.0	62.4	61.2	588.0	58.2
SD	2.5	2.3	4.8	7.1	2.3	23.6	4.1
P	.001	< .001	.612	0.186	< .001	.001	.002

For each individual histogram (n = 5–6; y-axis: number of events, x-axis: fluorescence intensity), eg, the average fluorescence intensity (Mean as column heading) and standard deviation (SD as column heading) has been calculated. In turn, these values were used to calculate the mean (Mean as row heading) and standard deviation (SD as row heading) of the means (column 1) or standard deviations (column 3), respectively, of all individual experiments.

Discussion

Paradoxically, erythrocytes from tg6 mice share features of young (increased intracellular potassium concentration, water content, flexibility, surface to volume ratio, and decreased cell density) as well as those of senescent (increased band 4.1a to 4.1b ratio, decreased GSH levels, reduced surface CD47 and sialic acids) erythrocytes. Presumably, tg6 erythrocytes age faster than normal ones resulting in enhanced phagocytosis of tg6 erythrocytes by wt and tg6 macrophages in vitro. This is reflected in vivo by the dramatically reduced erythrocyte life span in tg6 mice. In addition, tg6 macrophages showed an increased phagocytic activity when coincubated with both transgenic and wt erythrocytes. Moreover, in tg6 mice more macrophages per tissue area were found in the liver but less in the spleen. Considering that the transgenic liver and spleen are enlarged by 50% and 470%, respectively, tg6 mice have a drastically increased cell mass capable to incorporate erythrocytes. Together, these findings provide strong evidence for the existence of a so far unknown mechanism down-regulating the erythrocyte mass in excessive erythrocytosis most probably by enhancement of the normal physiological pathways to remove senescent erythrocytes. This includes first an adaptive increase in number and activity of tissue macrophages that remove erythrocytes and second accelerated erythrocyte aging that might be induced by oxidative damage as indicated by the lowered GSH levels. As a consequence, for example, CD47 loss of the whole cell population would facilitate erythro-phagocytosis in tg6. However, a reduction of CD47 concentration per surface area on the red cells could be in principle also be due to a gain of surface area after the erythrocytes have left the bone marrow. This has been observed in patients suffering from cirrhosis of the alcoholic[29] or in dogs after feeding with a cholesterol-rich diet[30] and is mediated by cholesterol incorporation into the erythrocyte membrane. This phenomenon could be confirmed in vitro by incubating red cells with cholesterol-rich lipid dispersions.[31] Indeed, our tg6 mice have a liver pathology since their liver is enlarged and shows hemosiderin dispositions and inflammatory foci—but tg6 mice do not show any signs of liver cirrhosis.[32] Of note, cholesterol incorporation into the red cell membrane leads to a decrease of osmotic resistance as well as erythrocyte flexibility.[30,31] However, as both parameters are increased in tg6 erythrocytes these observations argue against cholesterol overloading of their membrane. In addition, red cells of patients suffering from cirrhosis of the alcoholic show morphologic abnormalities such as target or spur cells[29,31] that we have never detected in blood smears of our tg6 mice (not shown). Finally, we have measured the CD47 content using FACS analysis. Compared with wt controls, these measurements revealed a reduced CD47 content per erythrocyte in tg6 mice. Since FACS analysis measures the antigen content per complete cell, it is independent on changes in membrane surface area due to lipid incorporation and a consecutive dilution of the membrane proteins. Therefore it is not very likely that a gain of membrane surface area due to lipid incorporation is responsible for the reduction of CD47 on the surface of tg6 erythrocytes. Whether recognition by macrophages is further enhanced by exposure of penultimate galactose residues or by opsonins remains open, since the negative Coombs test excludes the presence of induced autoantibodies, but cannot differentiate between unopsonized and opsonized cells by naturally occurring antibodies. On the other hand, phosphatidylserine exposure to the outer leaflet of the erythrocyte membrane, another age-related erythrocyte feature,[33,34] appears to be an important mechanism for red cell sequestration especially under pathological conditions such as sickle cell anemia since phosphatidylserine exposure is increased 2- to 10-fold in these patients.[4] However phosphatidylserine exposure did not differ significantly between wt and tg6 (not shown) indicating other mechanism(s) responsible for the reduced erythrocyte life span in tg6 mice.

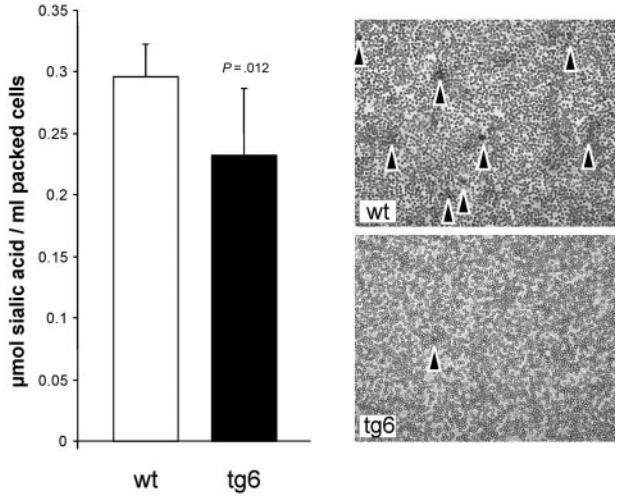


Figure 6. Erythrocyte surface sialic acids. Compared with wt, surface sialic acids were about 22% reduced in tg6 erythrocytes (left) despite the fact that the population of the transgenic erythrocytes is younger. The polybrene agglutination test (right) confirmed these quantitative measurements. Many red cell clusters (arrowheads) could be observed with wt erythrocytes, whereas hardly any could be detected with tg6 erythrocytes. Original magnification 200 ×; means (± SD) of 4 independent experiments each.

High hematocrit levels are found in patients suffering from chronic pulmonary failure, polycythemia vera, or chronic mountain disease, and in lowlanders at high altitude and in Epo-abusing athletes. Although humans rarely reach hematocrit values observed in our mice, there are cases described with hematocrit values up to

0.91.[35] Excessive erythrocytosis results in clinical complications such as hypertension, thromboembolism, and even death.[36]

As to the question how tg6 mice adapt to the extreme hematocrit values, we previously showed that compensatory mechanisms include vasodilatation and regulation of blood viscosity.[7,8,37] However vasodilatation leads to a decrease in blood flow velocity and thus fluid shear stress. Of note, as a non-Newtonian fluid, blood increases its viscosity with decreased shear stress.[38] Therefore, regulation of blood viscosity appears to be an at least as important mechanism as vasodilatation. The simplest way to maintain the blood viscosity as low as possible despite the elevated hematocrit is to keep the erythrocyte population as young as possible. Indeed, increased reticulocyte counts, a higher mean corpuscular volume, and somewhat lower (although not significant) mean corpuscular hemoglobin concentration in tg6 mice have been reported previously.[8] All these features characterize young erythrocytes.[39,40] The lower bumetanide-sensitive (NKCC) K^+ flux and higher ouabain-sensitive (active) K^+ flux also point to a younger erythrocyte population in tg6. This finding is in accordance with previous studies showing low basal NKCC activity and high Na/K-ATPase activity in reticulocytes that increases and decreases, respectively, with maturation.[41] The changes in the potassium transport kinetics we report here lead, via accumulation of intracellular K^+ and water, to an increased cell volume. The lower mean corpuscular hemoglobin concentration as a consequence of the accumulation of cations and water results in a lower internal viscosity and thus, together with the higher surface to volume ratio (especially in humans another feature of young erythrocytes[42]), to increased red cell flexibility as observed with osmotic gradient ectacytometry.

Under conditions of hypoxia-independent chronically elevated Epo plasma levels, as in our transgenic mice, so far unknown mechanism(s) might be activated to actively counteract the surplus of erythrocytes finally resulting in a markedly reduced erythrocyte life span. As shown here, one part of such a mechanism might be the increase in number and activity of tissue macrophages that are able to incorporate erythrocytes. If erythrocytes would be removed randomly whole blood viscosity would not change. In contrast, whole blood viscosity would decrease by an enhanced elimination of older erythrocytes that might be the second part of this mechanism. This possibility appears likely, although tg6 erythrocytes appear chronologically older than control cells based on the increased band 4.1a/4.1b ratio. Since even the lightest one third of the cells from tg6 mice have a higher band 4.1a/4.1b ratio (although not significant, not shown) than the lightest cells from controls, we speculate that tg6 erythrocytes remain longer in the bone marrow, but maintain their enzymatic set up until they enter the circulation.

In summary, our findings indicate the existence of a mechanism negatively regulating the erythrocyte mass that is capable to override the consequence of extreme plasma Epo levels.

Acknowledgments

This work was supported by Stiftung für wissenschaftliche Forschung an der Universität Zürich (J.V.); and Swiss National Science Foundation (3100B0-112449 and 3100A0-112216 to A.B. and M.G., respectively).

The authors thank S. Keller and B. Grenacher for technical support.

Authorship

Contribution: A.B. measured ion, water content, and surface sialic acids; D.M. measured ion fluxes and nonprotein thiols; H.L. performed osmoscans of CD47 and band 4.1 measurements and suggested article text revisions; B.S. performed band 4.1 measurements; M.G. generated the tg6 mice and revised article text and data presentation; J.V. designed the study, wrote the article, performed erythrocyte in vivo tracking, macrophage assay, polybrene testing and Coombs testing, and measured erythrocyte life span, osmotic fragility, and CD47.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

Correspondence: Johannes Vogel, Institute of Veterinary Physiology, Vetsuisse Faculty University of Zürich, Winterthurerstr. 260, CH-8057 Zürich, Switzerland; e-mail: jvogel@vetphys.uzh.ch.

References

1. Lutz HU. Innate immune and non-immune mediators of erythrocyte clearance. Cell Mol Biol. 2004; 50:107-116.
2. Burwell EL, Brickley BA, Finch CA. Erythrocyte life span in small animals; comparison of two methods employing radioiron. Am J Physiol. 1953;172:718-724.
3. Wandersee NJ, Tait JF, Barker JE. Erythroid phosphatidyl serine exposure is not predictive of thrombotic risk in mice with hemolytic anemia. Blood Cells Mol Dis. 2000;26:75-83.
4. Dasgupta SK, Thiagarajan P. The role of lactadherin in the phagocytosis of phosphatidylserine-expressing sickle red blood cells by macrophages. Haematologica. 2005;90:1267-1268.
5. Kiehne K, Schauer R. The influence of alpha- and beta-galactose residues and sialic acid O-acetyl groups of rat erythrocytes on the interaction with peritoneal macrophages. Biol Chem Hoppe Seyler. 1992;373:1117-1123.
6. Dale GL, Daniels RB. Quantitation of immunoglobulin associated with senescent erythrocytes from the rabbit. Blood. 1991;77:1096-1099.
7. Ruschitzka FT, Wenger RH, Stallmach T, et al. Nitric oxide prevents cardiovascular disease and determines survival in polyglobulic mice overexpressing erythropoietin. Proc Natl Acad Sci U S A. 2000;97:11609-11613.
8. Vogel J, Kiessling I, Heinicke K, et al. Transgenic mice overexpressing erythropoietin adapt to excessive erythrocytosis by regulating blood viscosity. Blood. 2003;102:2278-2284.
9. Wiessner C, Allegrini PR, Ekatodramis D, Jewell UR, Stallmach T, Gassmann M. Increased cerebral infarct volumes in polyglobulic mice overexpressing erythropoietin. J Cereb Blood Flow Metab. 2001;21:857-864.
10. Bogdanova AY, Ogunshola OO, Bauer C, Gassmann M. Pivotal role of reduced glutathione in oxygen-induced regulation of the Na(+)/K(+) pump in mouse erythrocyte membranes. J Membr Biol. 2003;195:33-42.
11. Lutz HU, Stammler P, Fasler S, Ingold M, Fehr J. Density separation of human red blood cells on self forming Percoll gradients: correlation with cell age. Biochim Biophys Acta. 1992;1116:1-10.
12. Hoffmann-Fezer G, Trastl C, Beisker W, et al. Preclinical evaluation of biotin labeling for red cell survival testing. Ann Hematol. 1997;74:231-238.
13. Schaller E, Macfarlane AJ, Rupec RA, Gordon S, McKnight AJ, Pfeffer K. Inactivation of the F4/80 glycoprotein in the mouse germ line. Mol Cell Biol. 2002;22:8035-8043.
14. Oldenborg PA, Zheleznyak A, Fang YF, Lagenaur CF, Gresham HD, Lindberg FP. Role of CD47 as a marker of self on red blood cells. Science. 2000;288:2051-2054.
15. Bratosin D, Mazurier J, Tissier JP, et al. Molecular mechanisms of erythrophagocytosis: characterization of the senescent erythrocytes that are phagocytized by macrophages. C R Acad Sci III. 1997;320:811-818.
16. Bratosin D, Mazurier J, Debray H, et al. Flow cytofluorimetric analysis of young and senescent human erythrocytes probed with lectins: evidence that sialic acids control their life span. Glycoconj J. 1995;12:258-267.
17. Blum H, Beier H, Gross HJ. Improved silver staining of plant proteins, RNA and DNA in polyacrylamide gels. Electrophoresis. 1987;8:93-99.
18. Aminoff D. Methods for the quantitative estimation of N-acetylneuraminic acid and their application to hydrolysates of sialomucoids. Biochem J. 1961;81:384-392.
19. Chambers DR, Procter J, Muratova O, et al. In vitro RBC exposure to Plasmodium falciparum has no effect on RBC antigen expression. Transfus Med. 2002;12:213-219.

20. Manodori AB, Kuypers FA. Altered red cell turnover in diabetic mice. J Lab Clin Med. 2002;140:161-165.
21. Steck TL. The organization of proteins in the human red blood cell membrane: a review. J Cell Biol. 1974;62:1-19.
22. Inaba M, Maede Y. Correlation between protein 4.1a/4.1b ratio and erythrocyte life span. Biochim Biophys Acta. 1988;944:256-264.
23. Frietsch T, Maurer MH, Vogel J, Gassmann M, Kuschinsky W, Waschke KF. Reduced cerebral blood flow but elevated cerebral glucose metabolic rate in erythropoietin overexpressing transgenic mice with excessive erythrocytosis. J Cereb Blood Flow Metab. 2007;27:469-476.
24. Oldenborg PA, Gresham HD, Lindberg FP. CD47-signal regulatory protein alpha (SIRPalpha) regulates Fcgamma and complement receptor-mediated phagocytosis. J Exp Med. 2001;193:855-862.
25. Bratosin D, Mazurier J, Tissier JP, et al. Cellular and molecular mechanisms of senescent erythrocyte phagocytosis by macrophages: a review. Biochimie. 1998;80:173-195.
26. Gattegno L. Changes in carbohydrate content of surface membranes of human erythrocytes during ageing. Carbohydr Res. 1976;52:197-208.
27. Gattegno L, Perret G, Fabia F, Cornillot P. Decrease of carbohydrate in membrane glycoproteins during human erythrocyte ageing in vivo. Mech Ageing Dev. 1981;16:205-219.
28. Gattegno L, Fabia F, Bladier D, Cornillot P. Physiological ageing of red blood cells and changes in membrane carbohydrates. Biomedicine. 1979;30:194-199.
29. Cooper RA, Diloy PM, Lando P, Greenverg MS. An analysis of lipoproteins, bile acids, and red cell membranes associated with target cells and spur cells in patients with liver disease. J Clin Invest. 1972;51:3182-3192.
30. Cooper RA, Leslie MH, Knight D, Detweiler DK. Red cell cholesterol enrichment and spur cell anemia in dogs fed a cholesterol-enriched atherogenic diet. J Lipid Res. 1980;21:1082-1089.
31. Cooper RA, Arner EC, Wiley JS, Shattil SJ. Modification of red cell membrane structure by cholesterol-rich lipid dispersions: a model for the primary spur cell defect. J Clin Invest. 1975;55:115-126.
32. Heinicke K, Baum O, Ogunshola OO, et al. Excessive erythrocytosis in adult mice overexpressing erythropoietin leads to hepatic, renal, neuronal, and muscular degeneration. Am J Physiol Regul Integr Comp Physiol. 2006;291:R947-R956.
33. Boas FE, Forman L, Beutler E. Phosphatidylserine exposure and red cell viability in red cell aging and in hemolytic anemia. Proc Natl Acad Sci U S A. 1998;95:3077-3081.
34. Lang F, Lang KS, Lang PA, Huber SM, Wieder T. Mechanisms and significance of eryptosis. Antioxid Redox Signal. 2006;8:1183-1192.
35. Jefferson JA, Escudero E, Hurtado ME, et al. Excessive erythrocytosis, chronic mountain sickness, and serum cobalt levels. Lancet. 2002;359:407-408.
36. Bertinieri G, Parati G, Ulian L, et al. Hemodilution reduces clinic and ambulatory blood pressure in polycythemic patients. Hypertension. 1998;31:848-853.
37. Quaschning T, Ruschitzka F, Stallmach T, et al. Erythropoietin-induced excessive erythrocytosis activates the tissue endothelin system in mice. Faseb J. 2003;17:259-261.
38. Bate H. Blood viscosity at different shear rates in capillary tubes. Biorheology. 1977;14:267-275.
39. Hill NS, Sardella GL, Ou LC. Reticulocytosis, increased mean red cell volume, and greater blood viscosity in altitude susceptible compared to altitude resistant rats. Respir Physiol. 1987;70:229-240.
40. Milligan DW, MacNamee R, Roberts BE, Davies JA. The influence of iron-deficient indices on whole blood viscosity in polycythaemia. Br J Haematol. 1982;50:467-471.
41. Mairbaurl H, Schulz S, Hoffman JF. Cation transport and cell volume changes in maturing rat reticulocytes. Am J Physiol Cell Physiol. 2000;279:C1621-C1630.
42. Waugh RE, Narla M, Jackson CW, Mueller TJ, Suzuki T, Dale GL. Rheologic properties of senescent erythrocytes: loss of surface area and volume with red blood cell age. Blood. 1992;79:1351-1358.

8.3 Paper 3 (manuscript in preparation)

Erythropoietin binding in the heart in vivo and in vitro: behind cardioprotection

*[1,2] Anna Bogdanova, [1,2]Deyan Mihov, [1]Nikolai Bogdanov, [1]Beat Grenacher, [1,2]Johannes Vogel, [1,2]Max Gassmann, [2,3]Reza Tavakoli

[1]Institute of Veterinary Physiology, University of Zurich, Zurich, Switzerland
[2]Zurich Center for Integrative Human Physiology (ZIHP), University of Zurich, Zurich, Switzerland
[3]Departments of Cardiovascular Surgery and Clinical Research, University Hospital Zurich, Zurich, Switzerland

Erythropoietin binding in the heart in vivo and in vitro: behind cardioprotection

*[1,2] Anna Bogdanova, [1,2]Deyan Mihov, [1]Nikolai Bogdanov, [1]Beat Grenacher, [1,2]Johannes Vogel, [1,2]Max Gassmann, [2,3]Reza Tavakoli

[1]Institute of Veterinary Physiology, University of Zurich, Zurich, Switzerland

[2]Zurich Center for Integrative Human Physiology (ZIHP), University of Zurich, Zurich, Switzerland

[3]Departments of Cardiovascular Surgery and Clinical Research, University Hospital Zurich, Zurich, Switzerland

Corresponding author:

Anna Bogdanova

Institute of Veterinary Physiology

Winterthurerstrasse 260

CH-8057 Zurich

Switzerland

Tel: +41 44 6358811

Fax: +41 44 6358932

E-mail: annab@access.uzh.ch

Number of words:

Abstract

Aims: Intravenous administration of human recombinant erythropoietin (Epo) was shown to reduce myocardial damage in various models of myocardial infarction and ischemia-reperfusion injury. This study was designed to characterize the targets of Epo action in the heart and the mechanisms of cardioprotection.

Methods: Heterotopic rat heart transplantation model and neonatal rat cardiomyocytes we have studied the pharmacokinetics of endogenous Epo and hrEpo in the heart and in plasma, localization of Epo binding, Epo-sensitive signaling pathways and its downstream targets.

Results: Our data indicate that Epo does not enter the myocardium when applied intravenously and its cardioprotective action is caused by the interaction with the coronary vessels. Neonatal and adult cardiomyocytes are also capable to bind hrEpo. Cytokine binding to the endothelial cells and cardiomyocytes occurs within 5-10 min and internalization and degradation of Epo-Epo receptor complex follows during 15-60 min. Interaction of hrEpo with the endothelial cells in vivo and with cardiomyocytes in vitro, results in transient activation of the downstream members of PI3K/Akt signaling pathway and increase in NO production during the first hour of Epo treatment and inhibition of the late inflammation-induced NO release. Early onset of eNOS activation by hrEpo causes reduction of the reperfusion-induced oxidative stress. Late activation of peroxynitrite production during inflammatory phase is inhibited by hrEpo.

Conclusions: Cardioprotective effect of Epo in vivo after the intravenous administration is mediated entirely by the factors secreted from the coronary vessels one of which is NO. Further studies are needed to characterize these cardioprotective factors.

Introduction

Cardioprotective potential of human recombinant erythropoietin (hrEpo) has been extensively studied during the past few years using in vivo models and isolated heart cells [1-9]. Despite numerous reports on the cytoprotective effects of Epo the underlying mechanisms including localization of the Epo binding, Epo-sensitive signaling pathways and downstream targets remain largely unknown.

The importance of the Epo-driven signaling in the myocardium during embryonic development has been convincingly demonstrated [10-12]. Localization of the EpoR in embryonic heart is a matter of discussion. Some reports suggest that it only is expressed in the endothelial cells of endocardium and in mesothelial cells of epicardium, but is missing in cardiomyocytes [11] whereas the other studies report the presence of EpoR in the embryonic myocardium [10, 12]. Knocking out EpoR is embryonic lethal and results in severe hypoplasia of the heart with concomitant reduction in number of cardiomyocytes and defects in cardiac morphogenesis [11]. Epo required for the stimulation of the myocyte proliferation is not generated by the myocytes themselves or delivered from plasma but is most likely released from the epicardium of the embryonic heart [10]. In adult heart, where cardiomyocytes are senescent, a paracrine Epo production has never been shown. Presence of EpoR in adult cardiac myocytes and fibroblasts was observed using immunohistochemical detection technique only [5]. However, particularly this approach was recently reported to give false readouts as none of the commercially produced antibodies for immunohistochemical EpoR detection were proven to recognize the receptor as shown by the isolation and subsequent characterization of the cross-reacting proteins using mass-spectrometry. The EpoR-positive staining obtained with the antibody used in most of the publications to actually prove the presence of EpoR in cardiomyocytes (M-20,

Santa Cruz) was particularly intensive in the myocardium of an $EpoR^{-/-}$ embryo [13]. This finding warrants re-evaluation of the data on the presence and localization of the Epo targets in the adult myocardium as the number of reports on the impressive cardioprotective potential grows exponentially during the last few years [5].

We have monitored the interaction of intravenously administered hrEpo with the heart in the native rat hearts as well as the hearts undergoing heterotopic heart transplantation, a model of cold transient global ischemia-reperfusion (I-R), to assess the stress-sensitivity of the cytokine binding. Epo binding capacity of cardiomyocytes was verified using the primary cultures of rat neonatal cells (NRCs). We assessed kinetics of Epo binding and the fate of EpoR-Epo complex thereafter for both experimental models as well as the Epo-induced responses of the PI3K/Akt signaling pathway and eNOS as a downstream target of this signaling cascade. We have then used the obtained data to analyze the possible mechanisms of Epo-induced cardioprotection against I-R injury in transplanted rat hearts [14].

Materials and methods

Animal handling and heterotopic heart transplantation

Male Lewis rats (250-300 g) were purchased from Harlan Laboratories, Holland and kept in the optimal sterile conditions in the animal facilities of the University Hospital Zurich. All the animals received human care in accordance with the European Convention on Animal Care and local institutional guidelines. Animals were blindly divided into two grous (donors and recipients). Of recipient animal were further divided into control and Epo-treated groups. Hearts of donor Lewis rats were arrested with cold crystalloid cardioplegia and after 45 min of cold global ischemia grafted heterotopically into the abdomen of recipient Lewis rats are described elsewhere [15]. Recipients were randomly

assigned to control non-treated or Epo-treated group receiving 5000 U/kg of rhEpo intravenously 20 min prior to reperfusion. At 5 time points (5 min, 30 min, 60 min, 6 h and 24 h) after reperfusion, the recipients (n=6-8 at each point) were sacrificed, blood and native and grafted hearts harvested for subsequent analysis

Cell isolation

Neonatal rat cardiomyocytes (NRCs) were isolated from the rat pups of postnatal days 3-4 as described elsewhere [16] and used for experiments within 5 days after isolation.

Quantification of the Epo levels in plasma and myocardial tissue

Plasma of donor and recipient rats was collected for detection of the endogenous rat Epo and hrEpo levels using ^{125}I-radioimmunoassay kit EPO-Trac^{TM} (DiaSorin, Stillwater, MI, USA). The same assay was used to detect endogenous and hrEpo levels in homogenates of ventricular tissue of native and transplanted blood-free hearts. Non-bound blood born hrEpo was removed by flushing coronary vessels and the ventricular chambers with ice-cold washing solution containing 300 mM sucrose and 20 mM Tris-HCl (pH 7.4).

Immunohistochemical hrEpo detection

Cryosections of the ventricular tissue of native and transplanted hearts were stained using mouse monoclonal anti-hrEpo antibodies (AMB2871, R&D Systems, 1:100 dilution) after fixation with 4% paraformaldehyde and permeabilisation with 0.3% solution of Triton X100 in PBS. Corresponding secondary antibodies for immunohistochemistry were obtained from Molecular Probes. The specificity of the staining was monitored by incubation of cryosections only with secondary antibody (Fig 1s) (goat anti-mouse). For visualization of blood vessel structure cryosections were incubated with antibodies against

Von Willebrand factor (Abcam, rabbit polyclonal ab6994) as a marker for endothelial cells, smooth muscle actin (#A2547, Sigma-Aldrich) as a marker of smooth muscle cell and myomesin (generated and kindly provided by Dr. Irina Agarkova) as a marker for cardiomyocytes (Fig 2s). NRCs were incubated with 10 U/ml hrEpo for 15 min to 3 h and subsequently fixed with 4% paraformaldehyde, blocked with 0.1M glycine and Triton X100-permeabilised. Goat polyclonal anti-Epo antibody (sc-1310, Santa Cruz Biotechnology Inc.) was used for immunocytochemical Epo detection.

^{125}I-Epo binding assay

^{125}I-Epo (Amersham) was used to assess kinetics of Epo interaction with plasma membrane of NRCs and its subsequent internalization. Kinetics of specific interaction of ^{125}I-Epo with the cells was assessed during 5 min to 3 h in the absence of non-radiolabeled hrEpo or in the presence of a 200x excess of non-radiolabeled hrEpo to quantify the non-specific binding of the tracer. After the incubation with ^{125}I-Epo radioactivity of the medium and cells was measured using a gamma-counter. Similar experiments were repeated with and without extra triple-washing in acetate buffer as described elsewhere [17] in order to discriminate between the membrane-bound and internalized ^{125}I-Epo.

Signal transduction pathways analysis

Phosphorylation state of Ser 473 of the Akt kinase was assessed in cryosections of the native and transplanted hearts of the recipient animals of control and Epo-treated groups using anti-phospho-Akt monoclonal rabbit antibody (#4058, Cell Signaling). Total and phosphorylated Akt forms were quantitatively detected in myocardial tissue homogenate using Elisa kits (R&D Systems). Nitric oxide synthase activation was assessed using rabbit polyclonal antibodies against eNOS phosphorylated at Ser 1177 (#9571, Cell Signaling).

The specificity of the staining was monitored by incubation of cryosections only with the secondary antibody (Fig 1s) (goat anti-rabbit). Epo-sensitive activation of the NO production in NRCs was monitored using chemiluminescent NO_2^- detection system (CLD-88 EcoMedics). Nitrate levels were assessed after reduction of NO_3^- to NO_2^- using conventional bi-metallic composition-based reduction reaction on copper-plated cadmium beads. Cells were incubated with 10 U/ml hrEpo for 30 min. Incubation medium was then collected and used for NO_2^-/NO_3^- detection along with cell lysates.

Results

Endogenous and hrEpo levels in plasma of healthy rats and those undergoing abdominal surgery

Endogenous erythropoietin plasma level in Lewis male rats was 86.1±2.3 mU/ml and rapidly declined even further by 40% in the recipient animals subjected to the heterotopic heart transplantation (Fig 1A). Plasma erythropoietin store remained depleted for the following 24 h after the operation. When 5000 U/kg hrEpo bolus was applied intravenously plasma levels went down from 130±7 U/ml immediately after the administration to 3.9±0.2 U/ml within 24 h, a level still 45-fold exceeding the basal one for the endogenous cytokine (Fig 1B). Half-life of hrEpo in rat plasma was calculated from the time course of the hrEpo clearance presented in Fig 1B. Double-exponential decay function was used for fitting in accordance with previous studies on the Epo pharmacokinetics in healthy rats and in human subjects [18]. The obtained $T_{1/2}$ of about 4.9 h was, although somewhat reduced, within the range obtained in healthy animals {Woo, 2007 #171}.

Epo binding in the myocardial tissue

Basal levels of endogenous Epo and binding of hrEpo to the myocardium was assessed quantitatively in homogenate of ventricular tissue using radioimmunoassay. Endogenous Epo content in homogenates of native hearts of recipients was low (0.12±0.04 mU/mg protein) and remained unaltered in both native and transplanted myocardium of the control animals group over the 24 h of reperfusion. Intravenous administration of hrEpo caused a rapid increase in hrEpo in both native and transplanted heart already within the first minutes of exposure (Fig 2). Acute Epo binding capacity did not differ for the native and transplanted hearts, but the clearance rate was 8.2-fold higher in the grafts compared to the native heart ($T_{1/2}$ of ~1.8 h in transplanted and that of ~15.2 h in native hearts, Fig 2). The possibility of contamination of the heart samples with the hrEpo originating from plasma could not be excluded despite thorough washing of blood from the coronary vessels with cold sucrose-Tris-HCl buffer. Due to this limitation these data were verified using an alternative approach. Localization of the hrEpo binding in the heart tissue was visualized by means of immunohistochemistry.

Interestingly, endogenous Epo binding to the coronary endothelium (see Fig 2s for the specific staining for endothelial, smooth muscle, and myocardial markers) of the grafted hearts but not the native ones could be observed 5 min after the onset of perfusion (Fig 3A, Fig 3s A). This observation is in line with a decrease in plasma endogenous Epo levels observed in the recipient animals (Fig 1A). As we have reported earlier [14] coronary vessels appear to be the main target for hrEpo binding in the heart over the first hour of Epo treatment (Fig 3 and Fig 3s). We were unable to detect hrEpo in the myocardium at any time point between 5 min and 24 h of perfusion (Fig 3B and Fig 3sB). In agreement with the data on the net hrEpo content in ventricular tissue homogenate (Fig 2) specific Epo staining was below detection limits in the hrEpo-treated grafts 6 h after the onset of perfusion. In the corresponding native heart it was still abundant (Fig 3B and Fig 3sB).

Both native and transplanted heart showed no specific Epo-positive staining 24 h after the transplantation (Fig 3B and Fig 3sB).

Epo binding and intracellular processing in NRCs

When added to the medium, hrEpo readily interacted with the NRCs (Figs 4 and 5). Kinetics of the hrEpo binding to the NRCs, its internalization and degradation were assessed by two independent techniques: radioactive tracer kinetics (^{125}I-hrEpo) and immunocytochemistry. Membrane-bound ^{125}I-Epo could be recognized by its ability to dissociate from the receptor at low pH (the quality of washing was controlled in a separate set of experiments) as previously reported for the erythroid precursor cells [17]. Specific binding of the radiolabeled Epo could be blocked by the presence of 100-200-fold excess of the non-radiolabeled Epo. Most of the cytokine remained membrane-bound for the first 15 min (Fig 4, 5B) with the subsequent internalization, sequestration in cistern-like compartments and degradation occurring within 30-60 min (Fig 4 and 5C, D). The majority of these cistern-like hrEpo deposits were shown to contain LAMP 1 protein indicating that these were lysosomes (data not shown). After 3 h of treatment cells were desensitized to the external hrEpo. The bound hrEpo pool was degraded and de novo binding did not occur despite its continuous presence of hrEpo in the cell culture medium.

Epo-induced signaling in the myocardium and in NRCs

Intravenous administration of rhEpo did not cause any detectible increase in phosphorylated Akt levels in ventricular tissue homogenate. In fact, a decrease in both total (Fig 6A) and phosphorylated (Fig 6B) forms of the Akt was detected in the transplanted as well as in native (data not shown) heart tissue in rats of both control and Epo-treated

groups from 30 min of reperfusion on. This delayed deprivation of the total Akt pool was less pronounced in Epo-treated animals although could not be prevented completely.

Immunohistochemical identification of the phosphorylated Akt in cryosections showed a transient up-regulation of Akt phosphorylation in coronary vessels (mainly endothelium) of the grafts and native hearts (Fig 6C). The activation of Akt could only be observed in the tissue exposed to hrEpo for 5-30 min in both transplanted and native hearts of the Epo-treated recipient animals (Fig 6C). Phosphorylation of Akt could be induced by treatment of NRCs with 10 U/ml of hrEpo for 30 min (Fig 7). Phosphorylation was transient appearing after 30 min (Fig 7C) of incubation during incubation with hrEpo.

One of the downstream targets of the activated Akt was the eNOS. Interestingly, intravenous hrEpo administration triggered acute activation of the eNOS in the coronary endothelium and smooth muscle layer of the grafts during the first 30 min of reperfusion (Fig 3B and Fig 3sB) and down-regulated delayed stimulation of the eNOS observed in control non-treated group during the 6-24 h of reperfusion (Fig 3A and Fig 3sA). This observation is in agreement with our previous findings on the nitrite levels in plasma of the transplanted animals [14]. As in the coronary vessels, eNOS was activated by the treatment of NRCs with hrEpo. The corresponding up-regulation of the nitrite/nitrate production was observed both under "normoxic" (20% O_2) and hypoxic (1% O_2) conditions, conditions where NO production is limited by the O_2 availability [19] (Table 1).

Discussion

Epo binding sites in the heart

The data we have obtained indicate that hrEpo may interact with several cell types in the heart including endothelial cells and neonatal cardiomyocytes. Characterization of these binding sites at the molecular level is ongoing. Kinetics of Epo interaction with NRCs and

coronary vessels, cytokine processing and the signaling pathways activated by Epo treatment share similarities with those described in erythroid precursor cells. Epo binding to both cardiomyocytes and endothelium occurs within minutes after exposure to the cytokine (Figs 2, 3 and 4). Formation of the Epo-receptor complex is followed by its internalization and degradation within 30-60 min in the NRCs and in the endothelium of transplanted myocardium (Fig 4 and 3). Binding is facilitated and processing of hrEpo is speeded up by about 8.2-fold in the endothelial cells of the grafted heart compared to that of the native hearts (Figs 2 and 3). These data are in good agreement with reports on kinetics of Epo interaction with erythroid precursors-derived cell lines Ba/F3 and UT-7 where most of the membrane-bound Epo was internalized and degraded within 1 h [17, 20]. According to our knowledge, all the data on Epo-receptor processing in cells have been obtained without applying any kind of stress. We are thus the first to report stress-induced sensitization of the coronary endothelium to endogenous as well as human recombinant Epo caused by I-R in our in vivo model (Figs 2 and 3). Degradation of the internalized Epo-receptor complexes was also markedly facilitated by stress (Fig 2).

Plasma Epo pharmacokinetics data (Fig 1) confirmed that endogenous plasma Epo store is rapidly depleted in the recipient animals undergoing heterotopic heart transplantation (Fig 1). The estimated half-life of hrEpo applied intravenously in the same recipient animals was 4.87 h. In plasma of young healthy rats $T_{1/2}$ for the hrEpo depended on the administered dose. It ranged from 4.8 to 8.8 h and being about 8 h for the hrEpo dose we have applied [18]. Thus hrEpo clearance in animals exposed to abdominal surgery exceeded that in healthy animals suggesting once more that not only binding but also clearance of Epo is stress-dependent. Values obtained for the T1/2 of human Epo in rats were close to those in humans (4-9 h, [21]) as sequences for Epo and EpoR share about 90% homology between these two species.

Activation of the IP3K/Akt signaling pathway in coronary endothelium (Fig 6C), HUVECs [22], cardiomyocytes (Fig 7) and erythroid precursor cells [23], [24] in response to Epo treatment is one more feature suggesting that Epo binding sites share similar features in all three cell types. Activation of this signaling cascade in response to Epo treatment in coronary vasculature and in isolated cardiomyocytes is acute and transient. This allows precise timing of the offset of the eNOS activation which is then followed by suppression o of the NO production at the later time points. Chronic activation of the IP3K/Akt pathway is reported to be deleterious due to the oxidative stress, dramatic increase of Foxo3a transcription protein and accumulation of its target transcripts [25].

Possible mechanisms of Epo-induced cardioprotection in the in vivo model

Earlier on we have assessed the cardioprotective potential of Epo in the same cold global I-R model [14]. Treatment of the recipient animals with 5000 U/kg hrEpo reduced myocardial damage and release of the cardiac troponin T during the early (30 min of reperfusion) and late (24 h of reperfusion) stages of the reperfusion injury. We have shown that the protective effect of hrEpo is not related to the reduction of apoptosis in the myocardial tissue but rather to the suppression of necrosis. The latter was largely attributed to the ability of Epo to prevent reperfusion-induced oxidative stress [14]. The present set of data adds to our understanding of the cardioprotective mechanisms. We have shown that treatment with Epo results in almost instantaneous activation of the eNOS within the coronary endothelium and smooth muscle layer. Activity of eNOS is suppressed in the grafts and in the native hearts during the late stages of reperfusion (6-24 h) in the Epo-treated group whereas in control group phosphorylation of eNOS at Ser 1177 is up-regulated indicating its activation. These data are in agreement with the changes in plasma NO_2^- levels in the recipient animals [14]. Late reperfusion time points in our model are

characterized by development of systemic inflammatory response that can be followed by the increased plasma IL-6 levels and high myeloperoxidase activity in the transplanted organs (Bogdanova and Tavakoli, unpublished data). Generation of the superoxide anion by activated neutrophils results in an increase in the circulating H_2O_2 levels and oxidative stress both in the myocardium and in the circulating erythrocytes [14]. In this highly oxidative environment up-regulation of the NO production would result in facilitated nitrosative stress [26]. Therefore, transient activation of eNOS during the early phase of reperfusion may improve restoration of blood perfusion in the transplanted organ by causing vasodilatation and at the same time reduce oxidative stress triggered by acute increase in oxygen availability as we have shown earlier [14]. Furthermore, Epo-induced activation of NO production supported rapid restoration of the ion/water balance in the graft. Efficient blood perfusion of the graft in Epo-treated recipient animals facilitated removal of the excessive interstitial fluid accumulated during ischemic period. Restoration of ion balance was further supported by Epo-induced activation of the Na,K-ATPase and suppression of the passive K^+ fluxes which we have observed in NRCs. Interestingly, in isolated cells the effect of the cytokine was most triggered by NO release (see Table1) since ion transporters were responding to Epo treatment only when L-arginine was present in the incubation medium [14]. These data indicate that Epo-induced regulation of the NO levels has a pleiotropic protective effect preserving both redox state and ion/water balance in the transplanted heart.

Taken together our findings suggest that cardioprotection induced by intravenous Epo administration is transient and may only be achieved in a relatively narrow time window supporting earlier reports on the time limitations of the cytokine cardioprotective efficiency [27]. Nitric oxide is most likely not the only cardioprotective factor released from the coronary endothelium in response to Epo treatment. Recent reports suggest that

endothelin-1 may be one of them [28]. Angiotensin, prostaglandin PGI2, VEGF, neuregulin, angiopoietin are the other potential candidates known to support signaling between the endothelium and myocardium [29]. These studies will provide the missing link between the observed cardioprotective action of Epo and the actual players involved in the reported effects and explain the controversies in the reported findings on the efficiency of Epo-induced cardioprotection.

Sources of Funding: This study was funded by the grants of the Zurich Center for Integrative Human Physiology and Swiss National Science Foundation (#112 449 to AB and # 116 433 to RT)

References

[1] Schwartzenberg S, Ben-Shoshan J, Keren G, George J. The role of erythropoietin in myocardial protection: potential mechanisms and applications. Expert Rev Cardiovasc Ther 2006;4:41-50.
[2] Gross ER, Gross GJ. Pharmacologic therapeutics for cardiac reperfusion injury. Expert Opin Emerg Drugs 2007;12:367-388.
[3] Bogoyevitch MA. An update on the cardiac effects of erythropoietin cardioprotection by erythropoietin and the lessons learnt from studies in neuroprotection. Cardiovasc Res 2004;63:208-216.
[4] Smith KJ, Bleyer AJ, Little WC, Sane DC. The cardiovascular effects of erythropoietin. Cardiovasc Res 2003;59:538-548.
[5] Riksen NP, Hausenloy DJ, Yellon DM. Erythropoietin: ready for prime-time cardioprotection. Trends Pharmacol Sci 2008;29:258-267.
[6] Arcasoy MO. The non-haematopoietic biological effects of erythropoietin. British journal of haematology 2008;141:14-31.
[7] Binbrek AS, Mittal B, Rao KN, Sobel BE. The potential of erythropoietin for conferring cardioprotection complementing reperfusion. Coron Artery Dis 2007;18:583-585.
[8] Westenbrink BD, Voors AA, Ruifrok WP, van Gilst WH, van Veldhuisen DJ. Therapeutic potential of erythropoietin in cardiovascular disease: erythropoiesis and beyond. Curr Heart Fail Rep 2007;4:127-133.
[9] Noguchi CT, Wang L, Rogers HM, Teng R, Jia Y. Survival and proliferative roles of erythropoietin beyond the erythroid lineage. Expert reviews in molecular medicine 2008;10:e36.
[10] Stuckmann I, Evans S, Lassar AB. Erythropoietin and retinoic acid, secreted from the epicardium, are required for cardiac myocyte proliferation. Dev Biol 2003;255:334-349.

[11] Wu H, Lee SH, Gao J, Liu X, Iruela-Arispe ML. Inactivation of erythropoietin leads to defects in cardiac morphogenesis. Development 1999;126:3597-3605.
[12] Yu X, Shacka JJ, Eells JB, Suarez-Quian C, Przygodzki RM, Beleslin-Cokic B*, et al.* Erythropoietin receptor signalling is required for normal brain development. Development 2002;129:505-516.
[13] Elliott S, Busse L, Bass MB, Lu H, Sarosi I, Sinclair AM*, et al.* Anti-Epo receptor antibodies do not predict Epo receptor expression. Blood 2006;107:1892-1895.
[14] Mihov D, Bogdanov N, Grenacher B, Gassmann M, Zund G, Bogdanova A*, et al.* Erythropietin protects from the reperfusion-induced myocardial oxidation by enhancing coronary endothelial nitric oxide production in vivo. Eur J Cardiothorac Surg 2009;in press.
[15] Ono K, Lindsey ES. Improved technique of heart transplantation in rats. J Thorac Cardiovasc Surg 1969;57:225-229.
[16] Schafer BW, Perriard JC. Intracellular targeting of isoproteins in muscle cytoarchitecture. J Cell Biol 1988;106:1161-1170.
[17] Walrafen P, Verdier F, Kadri Z, Chretien S, Lacombe C, Mayeux P. Both proteasomes and lysosomes degrade the activated erythropoietin receptor. Blood 2005;105:600-608.
[18] Woo S, Krzyzanski W, Jusko WJ. Target-mediated pharmacokinetic and pharmacodynamic model of recombinant human erythropoietin (rHuEPO). Journal of pharmacokinetics and pharmacodynamics 2007;34:849-868.
[19] Bogdanova A, Vogel J, Yakushev S, Segato M., Makhro A, Bogdanov N*, et al.* Hypoxic Heart: Be a Trout, not a Rat. In: Morris S, Vosloo A, eds. 4th CPB Meeting in Africa: MARA 2008. Maasai Mara Game Reserve, Kenya, July 19-25, 2008: Medimond International Proceedings, 2008:225-234.
[20] Gross AW, Lodish HF. Cellular trafficking and degradation of erythropoietin and novel erythropoiesis stimulating protein (NESP). The Journal of biological chemistry 2006;281:2024-2032.
[21] Fisher JW. Erythropoietin: physiology and pharmacology update. Experimental biology and medicine (Maywood, NJ 2003;228:1-14.
[22] Beleslin-Cokic BB, Cokic VP, Yu X, Weksler BB, Schechter AN, Noguchi CT. Erythropoietin and hypoxia stimulate erythropoietin receptor and nitric oxide production by endothelial cells. Blood 2004;104:2073-2080.
[23] Jelkmann W, Bohlius J, Hallek M, Sytkowski AJ. The erythropoietin receptor in normal and cancer tissues. Critical reviews in oncology/hematology 2008;67:39-61.
[24] Elliott S, Pham E, Macdougall IC. Erythropoietins: a common mechanism of action. Experimental hematology 2008;36:1573-1584.
[25] van Gorp AG, Pomeranz KM, Birkenkamp KU, Hui RC, Lam EW, Coffer PJ. Chronic protein kinase B (PKB/c-akt) activation leads to apoptosis induced by oxidative stress-mediated Foxo3a transcriptional up-regulation. Cancer research 2006;66:10760-10769.
[26] Pacher P, Beckman JS, Liaudet L. Nitric oxide and peroxynitrite in health and disease. Physiological reviews 2007;87:315-424.
[27] Moon C, Krawczyk M, Paik D, Lakatta EG, Talan MI. Cardioprotection by recombinant human erythropoietin following acute experimental myocardial infarction: dose response and therapeutic window. Cardiovasc Drugs Ther 2005;19:243-250.
[28] Piuhola J, Kerkela R, Keenan JI, Hampton MB, Richards M, Pemberton CJ. Direct cardiac actions of erythropoietin (EPO): effects on cardiac contractility, BNP secretion and ischemia-reperfusion injury. Clin Sci (Lond) 2007.

[29] Brutsaert DL. Cardiac endothelial-myocardial signaling: its role in cardiac growth, contractile performance, and rhythmicity. Physiological reviews 2003;83:59-115.

Figure legends

Fig 1 Plasma endogenous (A) and hrEpo (B) levels. A: Donor plasma endogenous Epo levels were chosen as basal (time zero, N=35) and kinetics of the changes monitored in the recipient animals of the control group undergoing 5 min, 1 h and 24 h reperfusion (means ±SEM, N=6-8 foe each time point). * denotes $p<0.05$ and *** stands for $p<0.0001$ compared to the basal level B: Changes in the hrEpo levels in plasma of the Epo-treated recipient animals 5 min, 30 min, 1 h, 6 h or 24 h after the onset of reperfusion (means ±SEM, N=6-8 foe each time point). Curve fit was performed using an exponential decay function $y = 8.9+122.9e^{-0.008x}$, R=0.996.

Fig 2 Changes in the Epo content in crude homogenates of the ventricular tissue of native and transplanted hearts (means ±SEM, N=6-8 foe each time point). Numbers represent half-life of hrEpo in the native and transplanted heart tissue.

Fig 3 Immunostaining of cryosections from transplanted hearts of control animals (A) and rats treated with 5000 U/kg rhEpo (B). Cryosections from different reperfusion time points (5, 30, 60, 360, 1440 min) were stained with antibodies against Epo (red channel, middle columns) and phospho-eNOS at Ser 1177 (green channel, right columns). Nuclei were visualized with DAPI fluorescent stain (blue channel). The corresponding merged pictures (bright field + DAPI + Epo + phospho-eNOS) are presented on the left side of the panels A and B. Faint signal form endogenous Epo binding in graft of the control recipient animal (A, white arrows) was observed 5 min after the onset of reperfusion. The specific staining for Epo was observed solely in coronary vessels (A and B) with no staining detected in the cardiomyocytes at any reperfusion time point.

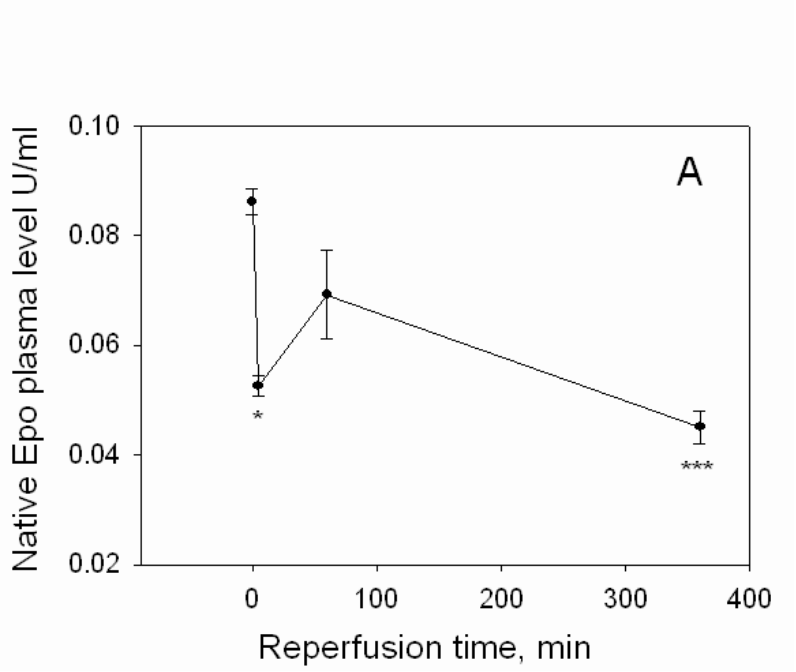
Fig 4 Specific interaction of ^{125}I-Epo with primary cultures of neonatal rat cardiomyocytes (NRCs). Presented are means ±SEM of 5 independent experiments where the amount of ^{125}I-Epo bound to the membrane (▲) and internalized fraction (o) was differentially assessed along with total binding (•).

Fig 5 Subcellular localization of the bound Epo visualized with fluorescent microscopy. Presented are non-treated control cells (A), NRCs 15 min x100 (B) and 30 min after Epo administration at x100 (C) and x40 (D) magnification.

Fig 6 The amount of total (A) and phosphorylated (B) Akt in crude homogenate of control and Epo-treated grafts at different time points after the onset of perfusion. Data are means ±SEM of 6-8 idependent experiments. * represents $p<0.05$ compared to the levels in donor hearts (time zero), # denotes $p<0.05$ between the corresponding non-treated and Epo-treated samples. (C) Immunostaining of cryosections (5 and 30 min reperfusion time points) with antibody against phosphorylated Akt at Ser 473. The specific fluorescent signal (green channel) is presented on the right side of the panels and the corresponding merged picture (bright field + phospho-Akt) on the left.

Fig 7 NRCs immunostaining for the phospho-Akt. Specific staining for the myomesin is shown in red, DAPI nuclear staining is in blue and that for the phospho-Akt in green. Negative control (A), 15 min (B), 30 min (C) or 3 h (D) of treatment with 10 U/ml hrEpo.

Fig 1


A
Native Epo plasma level U/ml
0.10
0.08
0.06
0.04
0.02
*
***
0
100
200
300
400
Reperfusion time, min

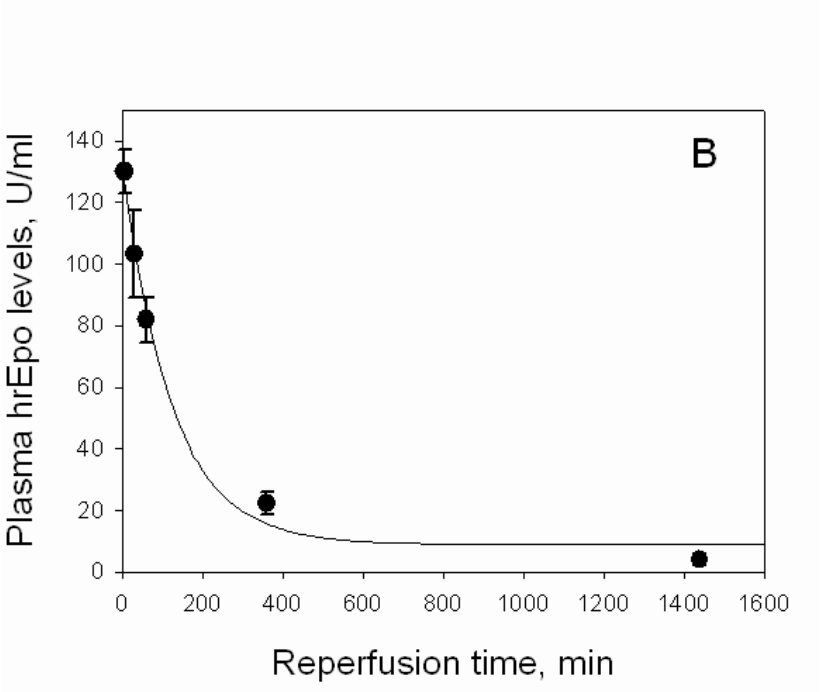

B
Plasma hrEpo levels, U/ml
140
120
100
80
60
40
20
0
0
200
400
600
800
1000
1200
1400
1600
Reperfusion time, min

Fig 2

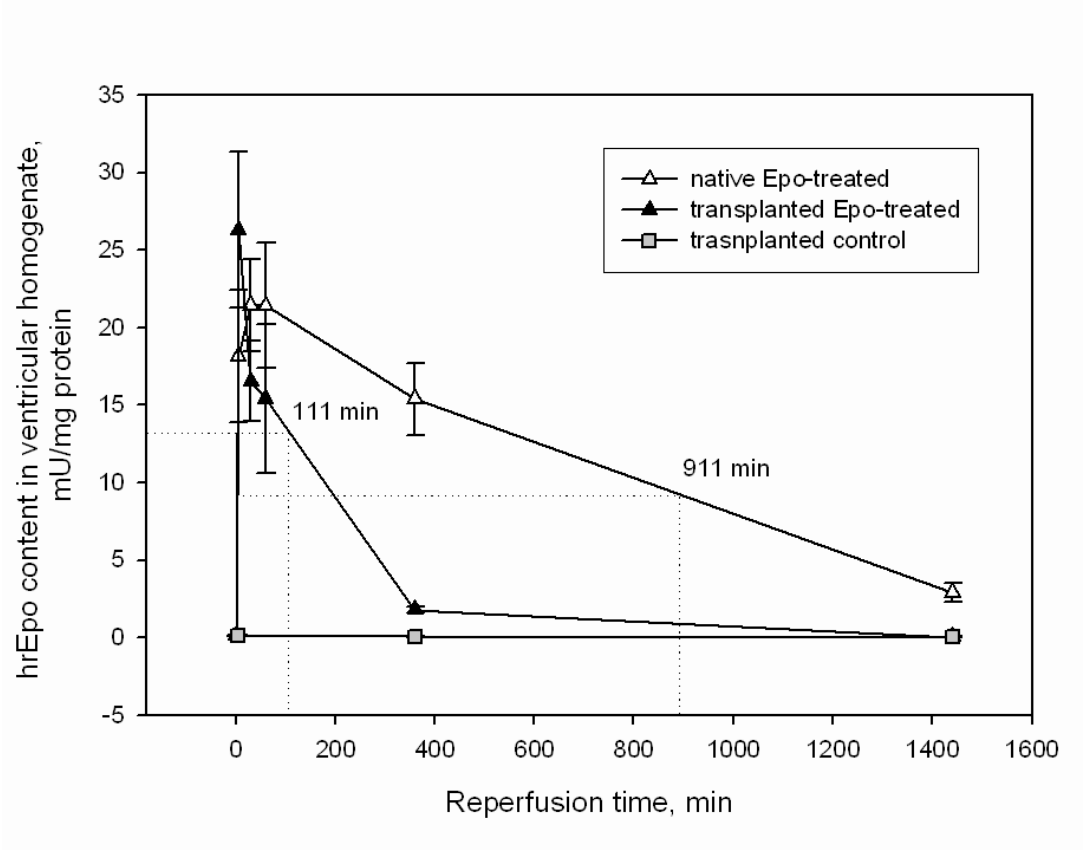

native Epo-treated
transplanted Epo-treated
trasnplanted control
111 min
911 min
hrEpo content in ventricular homogenate, mU/mg protein
Reperfusion time, min
35
30
25
20
15
10
5
0
-5
0
200
400
600
800
1000
1200
1400
1600

Fig 3 Cardiac tissue binging (5, 60, 360 and 1440 min)

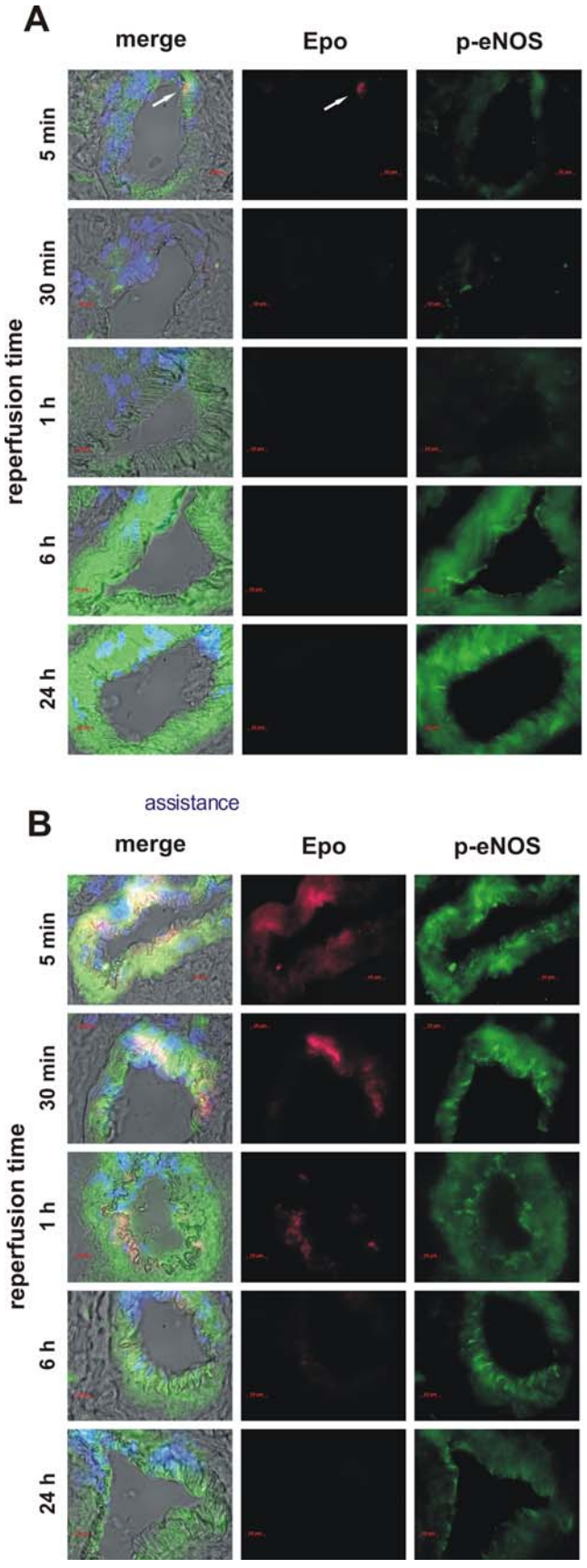


Fig 4

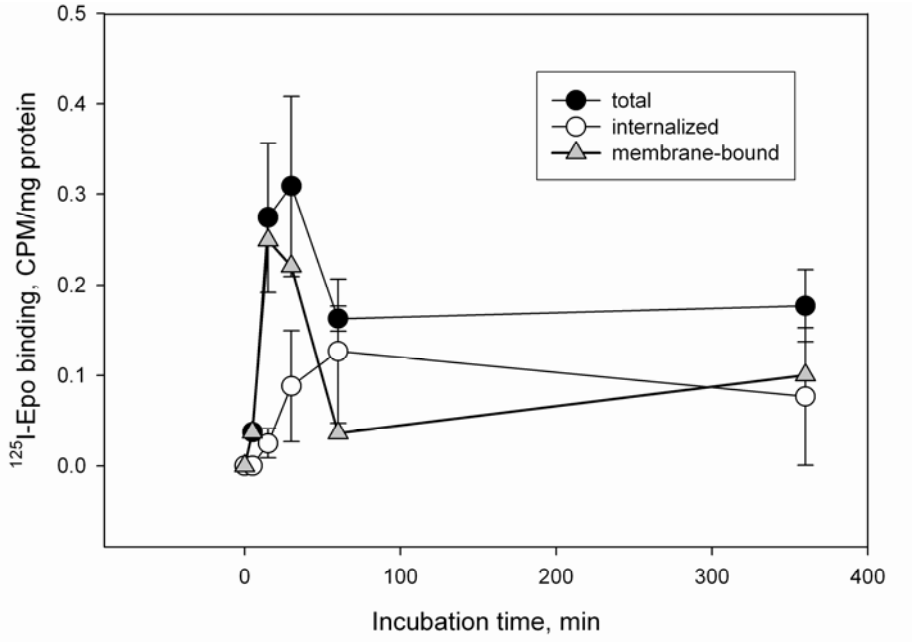

0.5
0.4
0.3
0.2
0.1
0.0
^{125}I-Epo binding, CPM/mg protein
total
internalized
membrane-bound
0
100
200
300
400
Incubation time, min

Fig 5

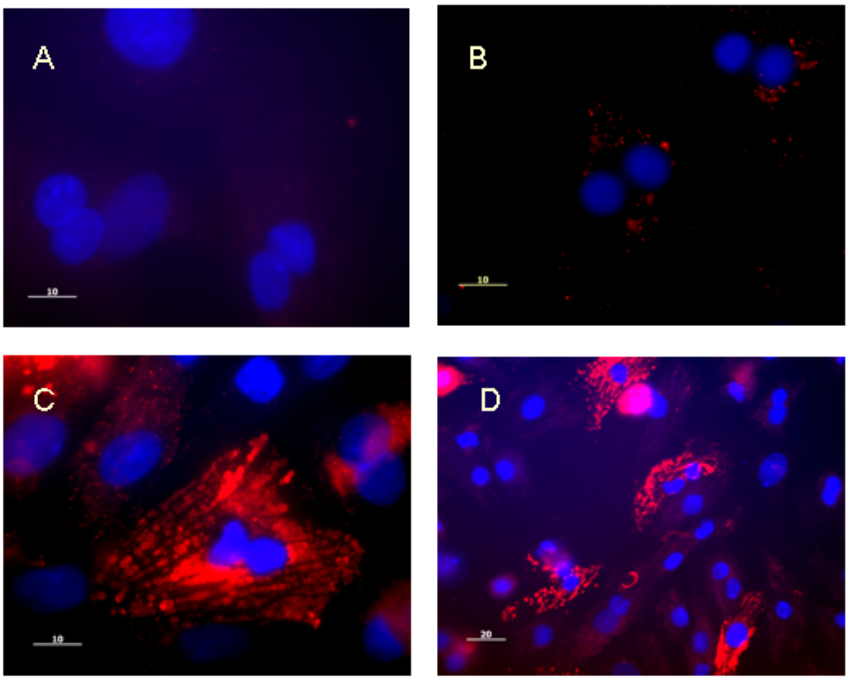

A
B
C
D

Fig 6, A and B

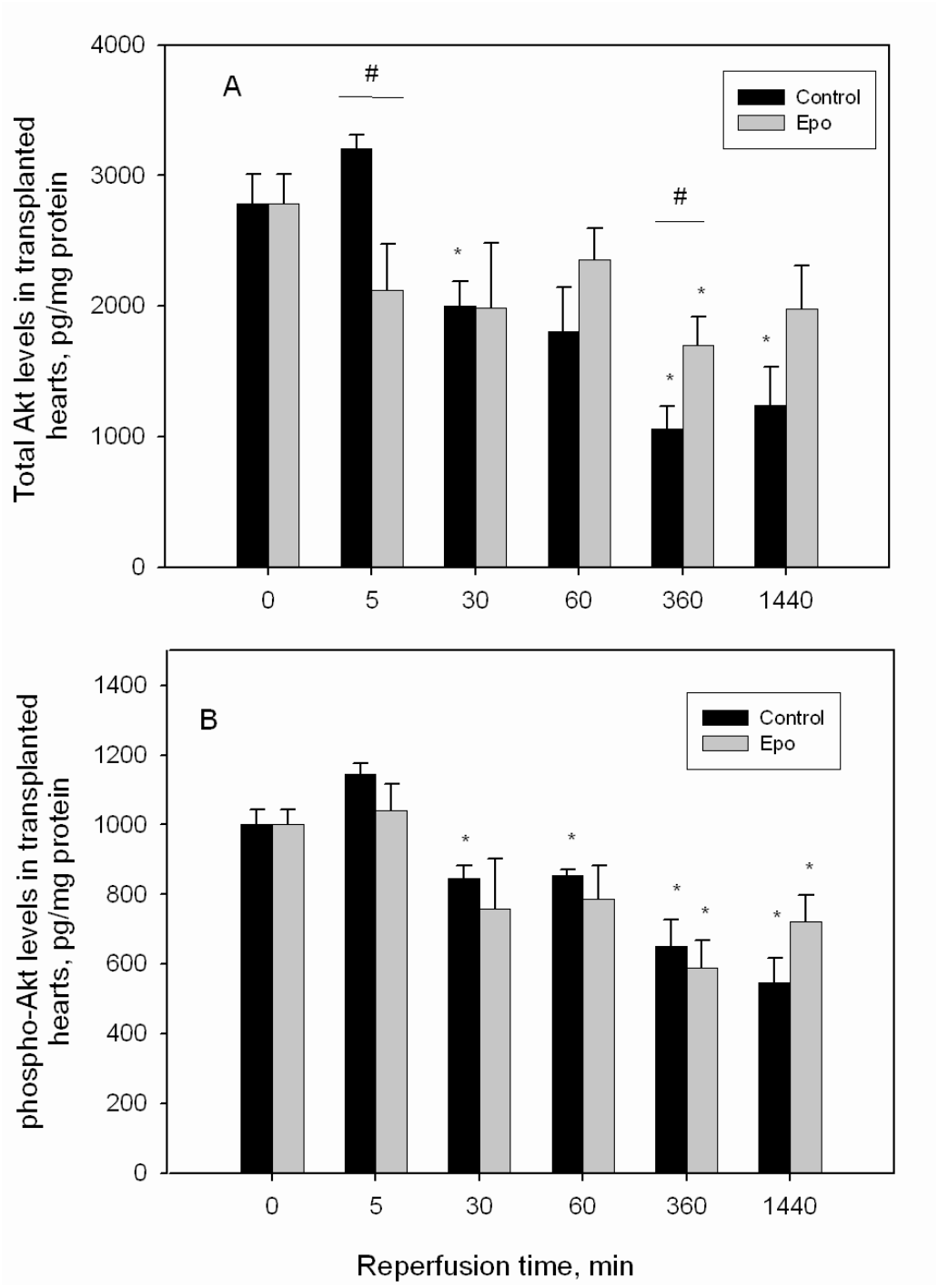

A
B
Control
Epo
Total Akt levels in transplanted hearts, pg/mg protein
phospho-Akt levels in transplanted hearts, pg/mg protein
Reperfusion time, min
0
5
30
60
360
1440
#
*

Fig 6 C

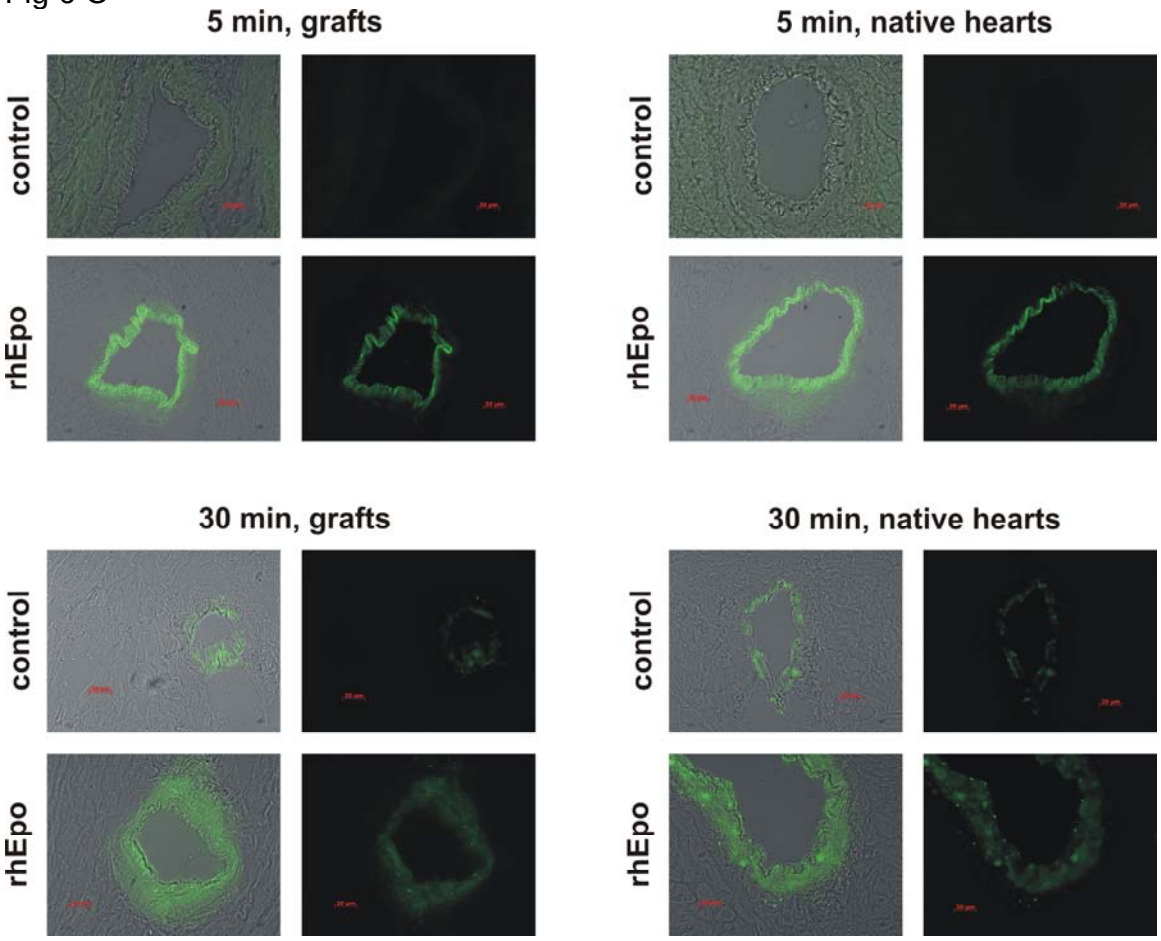

5 min, grafts
5 min, native hearts
30 min, grafts
30 min, native hearts
control
rhEpo
control
rhEpo
control
rhEpo
control
rhEpo

Fig 7

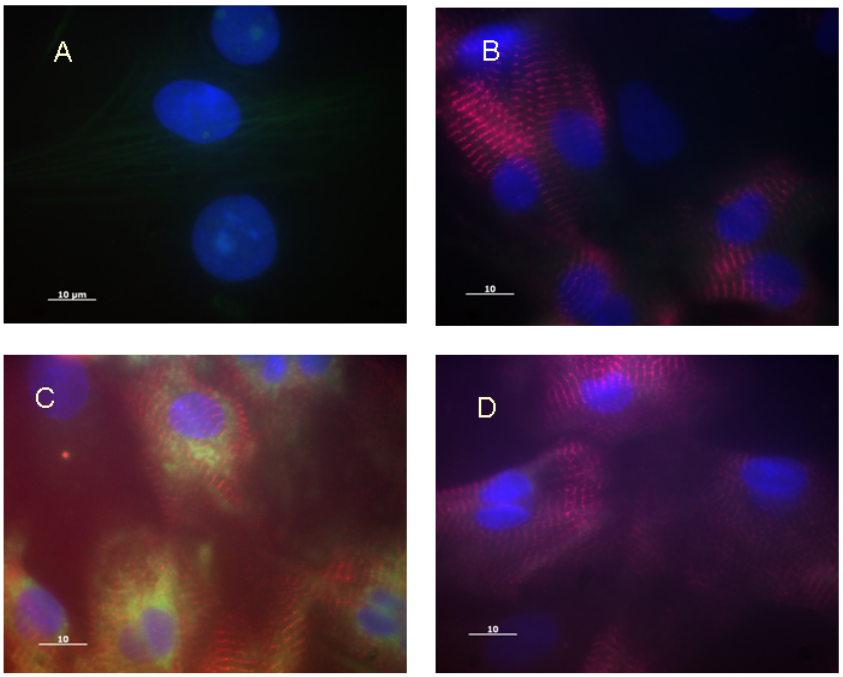

A
B
C
D
10 µm
10
10
10

Table 1

	Normoxia	Hypoxia
NO_2^-		
control	100	87.5±2.8*
Epo	104.9±8.8	179.1±29.5*
NO_3^-		
control	100	102.8±12.1
Epo	127.8±10.9*	122.5±9.4*

Supplemental files

Fig 1s
Negative control for the non-specific binding of the secondary antibodies used to visualize Epo and p-eNOS localization. Shown in panel 1 is a bright filed image overlayed with DAPI staining of the nuclei (shown in blue) as well as the staining in red and green fluorescent channels as in Figs 3 and 3s. Panel 2 shows fluorescent signal in red channel (used for Epo staining) and panel 3 shows fluorescence reordered in the green channel (p-eNOS visualization).

Fig 2s
Staining of the cryosections with specific markers of endothelial cells (von Willdebrand factor, shown in yellow), smooth muscle layer (sm actin, shown in red) and cardiomyocytes (myomesin, shown in green). Panels A1 and B1 show the overlay of all three channels with DAPI staining of the nuclei (shown in blue) and the bright field image for in the coronary vessel (A) and in the myocardium (B). Panels 2-4 represent the corresponding individual fluorescent channel readouts.

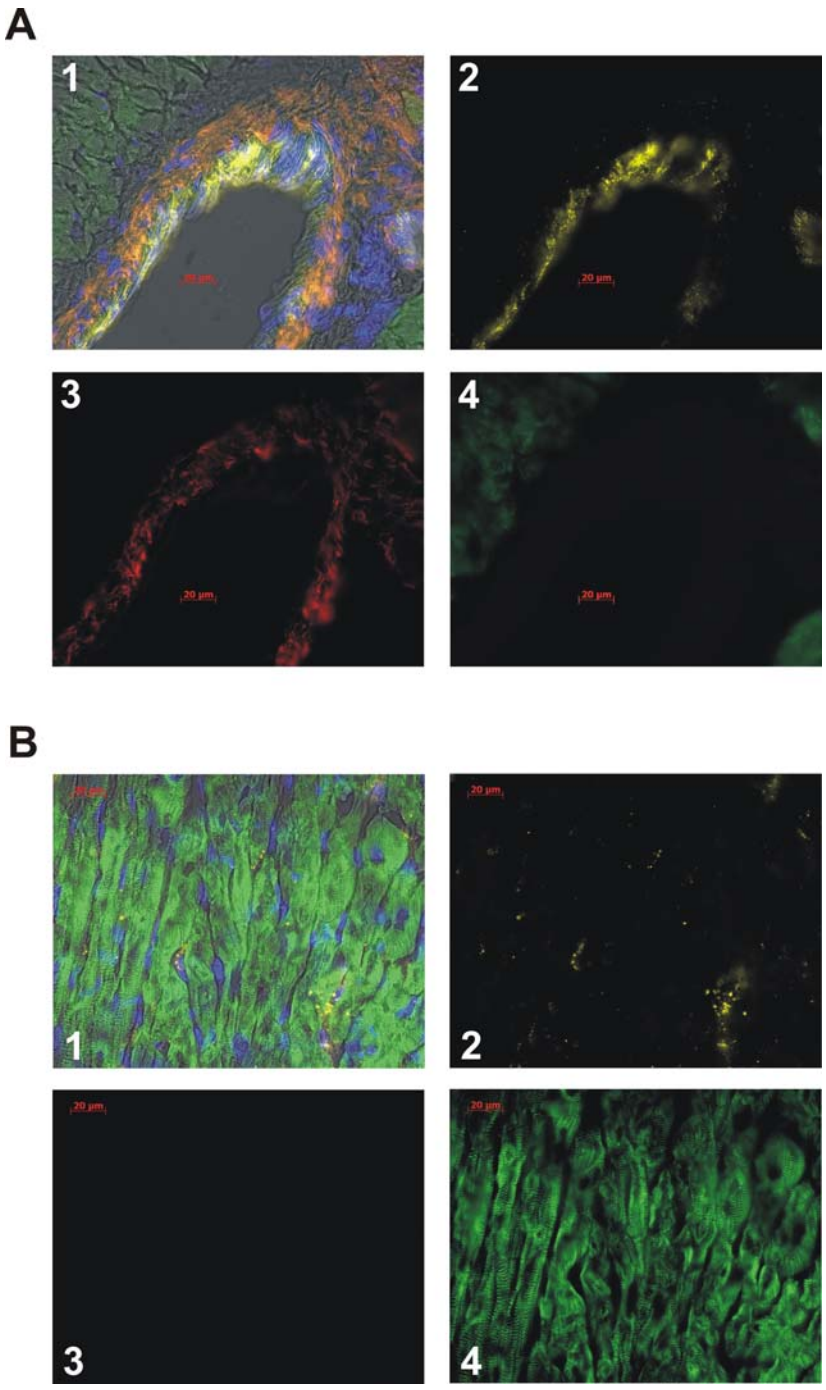
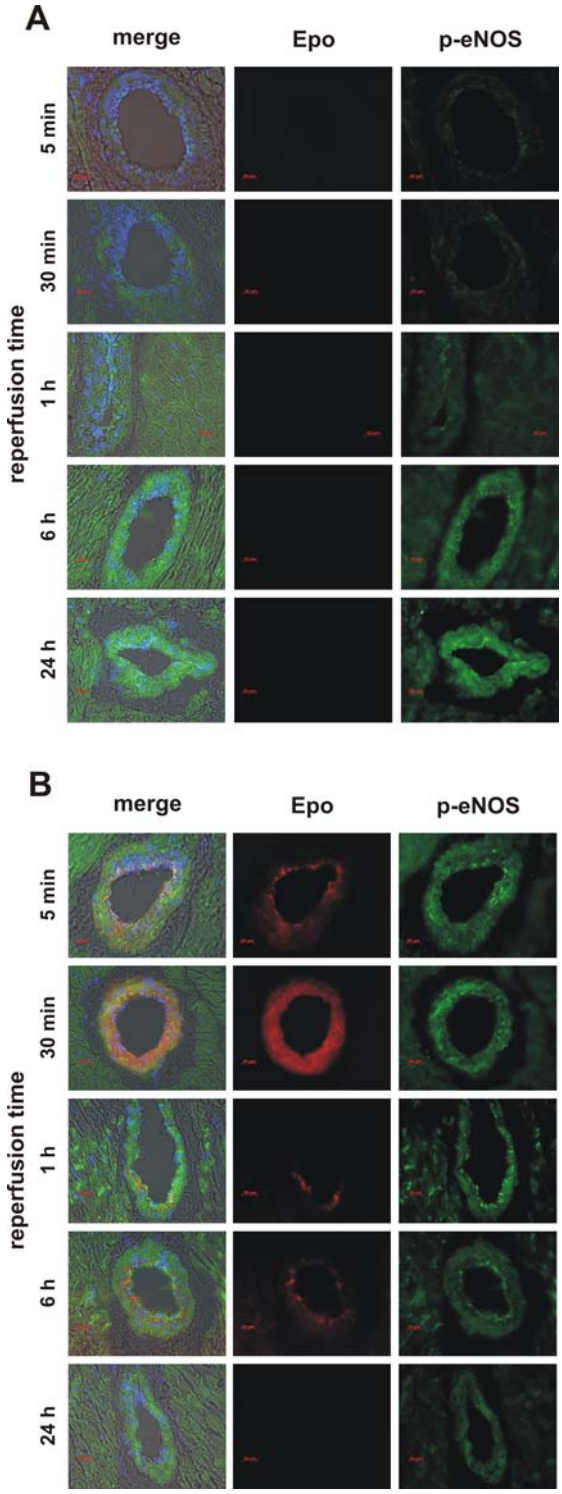


Fig 3s
Localization of Epo binding and eNOS phosphorylation in native hearts of control (A) and Epo-treated (B) recipient animals. Cryosections were stained with the antibodies against Epo (red channel) and phosphorylated eNOS (p-Ser 1177, green channel) in the native hearts of control (A) and Epo-treated (B) animals. Nuclei stained with DAPI are shown in blue.



8.4 Paper 4 (published manuscript)

Erythropoietin protects from reperfusion-induced myocardial injury by enhancing coronary endothelial nitric oxide production

Deyan Mihov, Nikolay Bogdanov, Beat Grenacher, Max Gassmann, Gregor Zünd, Anna Bogdanova, Reza Tavakoli

Eur J Cardiothorac Surg (2009), doi:10.1016/j.ejcts.2008.12.049
Published online [ahead of print] on 21 February 2009

ELSEVIER
European Journal of Cardio-thoracic Surgery xxx (2009) xxx–xxx
EUROPEAN JOURNAL OF CARDIO-THORACIC SURGERY
www.elsevier.com/locate/ejcts

Erythropoietin protects from reperfusion-induced myocardial injury by enhancing coronary endothelial nitric oxide production☆,☆☆

Deyan Mihov[c], Nikolay Bogdanov[c], Beat Grenacher[c], Max Gassmann[c,d], Gregor Zünd[b], Anna Bogdanova[c,d,1], Reza Tavakoli[a,b,d,1,*]

[a] Department of Cardiovascular Surgery, University Hospital Zurich, Switzerland
[b] Department of Clinical Research, University Hospital Zurich, Switzerland
[c] Institute for Veterinary Physiology, Vetsuisse Faculty, Switzerland
[d] Center for Integrative Human Physiology, University of Zurich, Switzerland

Received 19 September 2008; received in revised form 18 December 2008; accepted 22 December 2008

Abstract

Objective: Cardioprotective properties of recombinant human Erythropoietin (rhEpo) have been shown in in vivo regional or ex vivo global models of ischemia–reperfusion (I/R) injury. The aim of this study was to characterize the cardioprotective potential of rhEPO in an in vivo experimental model of global I/R approximating the clinical cardiac surgical setting and to gain insights into the myocardial binding sites of rhEpo and the mechanism involved in its cardioprotective effect. **Methods:** Hearts of donor Lewis rats were arrested with cold crystalloid cardioplegia and after 45 min of cold global ischemia grafted heterotopically into the abdomen of recipient Lewis rats. Recipients were randomly assigned to control non-treated or Epo-treated group receiving 5000 U/kg of rhEpo intravenously 20 min prior to reperfusion. At 5 time points 5–1440 min after reperfusion, the recipients (n = 6–8 at each point) were sacrificed, blood and native and grafted hearts harvested for subsequent analysis. **Results:** Treatment with rhEpo resulted in a significant reduction in myocardial I/R injury (plasma troponin T) in correlation with preservation of the myocardial redox state (reduced glutathione). The extent of apoptosis (activity of caspase 3 and caspase 9, TUNEL test) in our model was very modest and not significantly affected by rhEpo. Immunostaining of the heart tissue with anti-Epo antibodies showed an exclusive binding of rhEpo to the coronary endothelium with no binding of rhEpo to cardiomyocytes. Administration of rhEpo resulted in a significant increase in nitric oxide (NO) production assessed by plasma nitrite levels. Immunostaining of heart tissue with anti-phospho-eNOS antibodies showed that after binding to the coronary endothelium, rhEpo increased the phosphorylation and thus activation of endothelial nitric oxide synthase (eNOS) in coronary vessels. There was no activation of eNOS in cardiomyocytes. **Conclusions:** Intravenous administration of rhEpo protects the heart against cold global I/R. Apoptosis does not seem to play a major role in the process of tissue injury in this model. After binding to the coronary endothelium, rhEpo enhances NO production by phosphorylation and thus activation of eNOS in coronary vessels. Our results suggest that cardioprotective properties of rhEpo are at least partially mediated by NO released by the coronary endothelium.

Keywords: Erythropoietin; Myocardial ischemia–reperfusion injury

1. Introduction

Myocardial protection from ischemia and reperfusion (I/R) injury remains of paramount importance in the setting of global myocardial ischemia associated with open-heart surgical procedures. There has been considerable progress made to date in myocardial protection strategies including the use of blood cardioplegia [1]. Nevertheless, a high risk subset of patients continue to suffer perioperatively from myocardial I/R injury as exhibited by prolonged contractile dysfunction, low-output syndrome, arrhythmias, perioperative myocardial infarction and cardiac failure leading to prolonged intensive care [2]. Thus, there is a compelling necessity to improve myocardial protection from I/R injury during open-heart surgical procedures.

There is a large body of experimental work showing the non-hematopoietic cytoprotective properties of recombinant human Erythropoietin (rhEPO) in a variety of tissues subjected to I/R [3], including the retina [4], brain [5], and cardiovascular system [6]. However, primary targets and down stream mechanisms of the cardioprotective effect of rhEpo remain controversial. The aim of this study was to

☆ Presented at the 22nd Annual Meeting of the European Association for Cardio-thoracic Surgery, Lisbon, Portugal, September 14–17, 2008.
☆☆ This study was funded by the Zurich Centre for Integrative Human Physiology for RT and AB and the Swiss National Science Foundation grants (#112 449 for AB).
* Corresponding author. Address: Department of Thoracic and Cardiovascular Surgery, Canton Hospital Lucerne, 6000 Lucerne 16, Switzerland. Tel.: +41 41 205 45 02; fax: +41 41 205 45 63.
E-mail address: reza.tavakoli@ksl.ch (R. Tavakoli).
[1] These authors contributed equally to this work.

1010-7940/$ – see front matter
doi:10.1016/j.ejcts.2008.12.049

characterize the cardioprotective effect of rhEPO in an in vivo model of cold global I/R injury and to gain insights into the primary targets and down stream mechanisms involved in its cardioprotective effect.

2. Materials and methods

2.1. Animals

Male Lewis rats (Harlan, Holland) weighing 250–300 g were used as donors and recipients. All animals received humane care in compliance with the European Convention on Animal Care. This study was approved by the local institutional ethics committee. The animals were maintained in standard housing conditions with dry diet and water available ad libitum.

2.2. Heterotopic heart transplantation

Donor rats were anesthetized with an i.p. injection of pentobarbital (50 mg/kg of body weight) followed by administration of 500 units of i.v. heparin. The heart was arrested with 10 ml/kg of body weight of cold (4 °C) crystalloid cardioplegic solution and stored in the same cardioplegic solution at 4 °C until implantation.

Recipient rats were anesthetized with an i.p. injection of pentobarbital and the donor heart was transplanted heterotopically according to the technique of Ono and Lindsey [7]. The total duration of global ischemia was 45 min of which the last 15 min were needed for anastomoses.

2.3. Experimental design

Recipient rats were randomized to control non-treated or Epo-treated group receiving 5000 U/kg of rhEpo intravenously 20 min prior to reperfusion. At 5 time points (5, 30, 60, 360, and 1440 min) after reperfusion the recipients ($n = 6–8$ at each point) were sacrificed. Blood, native and grafted hearts were harvested for subsequent analyses.

2.4. Myocardial injury and tissue stress markers

Myocardial injury was assayed by monitoring of the plasma levels of cardiac troponin T isoform (cTnT) using electrochemiluminescent immunoassay (Roche Diagnostics, Switzerland). Plasma levels of atrial natriuretic peptide (ANP) and brain natriuretic peptide (BNP) were determined by enzymatic immunoassay (Peninsula Laboratories, USA).

2.5. Systemic inflammatory response

Interleukin-6 (IL-6) in blood plasma was assessed using Rat IL-6 Quantikine ELISA Kit (R&D Systems, Minneapolis, MN).

2.6. Measurement of tissue ion and water content

Tissue Na^+ and water content were assessed after drying and burning the tissue sampled in metal-free concentrated HNO_3 using flame photometry and gravimetry, respectively.

2.7. Apoptosis markers and signaling

Caspase 3 and caspase 9 activities were determined in tissue homogenate using colorimetric peptide-based assay kits (R&D Systems). Lysates of rat endothelial cell line (RBE4) treated overnight with 0.5 μM Staurosporine (Sigma–Aldrich, St. Louis, MO) were used as a positive control of apoptosis [8]. The downstream product of active caspase 3, cleaved poly (ADP-ribose) polymerase (PARP) was detected using immuno-blotting with the rat-specific (Asp214) rabbit polyclonal antibody diluted 1:1000 (Cell Signaling). The CardioTACS in situ apoptosis detection kit (R&D Systems) was used to estimate the number of TUNEL-based apoptosis-positive nuclei.

2.8. Red cell, plasma and tissue reduced (GSH)/oxidized Glutathione (GSSG) measurement

Blood and plasma samples were deproteinized and GSH and GSSG were detected using Ellman's reagent as described elsewhere [9]. Tissue samples used for determination of the GSH/GSSH levels were homogenized in equal volumes of solutions containing either 100 mM KCl and 10 mM MOPS-KOH or 5% trichloroacetic acid (TCA) in distilled water. After centrifugation supernatant was used for the GSH/GSSG detection using the same protocol as for the blood/plasma samples (see above).

2.9. Product of nitric oxide (NO) metabolism in plasma

Stable product of NO oxidation, i.e. plasma nitrite level, was detected using chemiluminescence assay by means of CLD-88 (EcoMedica, Switzerland). Plasma samples were collected immediately after blood harvest and concomitant centrifugation (4 min, 8000 g).

2.10. Epo-induced signaling

Binding of rhEpo was assessed in acetone-fixed cryosections of transplanted and native ventricular tissue using antibodies against Epo (R&D Systems).

Rabbit polyclonal antibodies against phosphorylated (Ser 1177) endothelial nitric oxide synthase (eNOS) (Cell Signaling, 1:100) were used for immunohistochemical determination of eNOS phosphorylated eNOS in acetone-fixed frozen sections. Secondary FITC-conjugated antibodies were applied and localization of the specific staining determined using fluorescent microscopy (Zeiss AxioScope 2500).

2.11. K^+ influx measurements in neonatal rat cardiomyocytes

The procedure of cell isolation is described in detail elsewhere [10]. Unidirectional K^+ influx was measured using radioactive tracer technique. The cells (≈400 000 cells per Petri dish, 3 cm ∅) were pre-treated with 1 mM ouabain and/or rhEpo (final activity 10 U/ml) in the presence or absence of 100 μM L-arginine for 30 min. Thereafter flux detection was initiated by adding ^{86}Rb as a tracer for K^+ (final activity 0.5 μCi/ml). Fluxes were assessed over half an hour with tracer accumulation detected 5, 10, 20, and 30 min after the

Please cite this article in press as: Mihov D, et al. Erythropoietin protects from reperfusion-induced myocardial injury by enhancing coronary endothelial nitric oxide production. Eur J Cardiothorac Surg (2009), doi:10.1016/j.ejcts.2008.12.049

tracer application. Over this time period the accumulation of tracer was a linear function of the incubation time. Samples (10 μl) of the incubation media were collected and the cells were washed free from extracellular ^{86}Rb with ice-cold incubation medium and finally lysed in 0.1 M NaOH. Cell lysate was then used for detection of the intracellular ^{86}Rb and protein assessment (Bio-Rad protein assay). Unidirectional potassium influx was then calculated from the slope of the ^{86}Rb uptake curve over time and normalized per amount of the ^{86}Rb in the incubation medium and per protein. Active K^+ transport mediated by the Na/K ATPase was calculated as a difference between K^+ fluxes in the presence and in the absence of ouabain. Ouabain-resistant flux component was addressed to as passive K^+ influx.

2.12. Statistical analysis

All data are based on at least six replicates for in vivo experiments and at least five independent experiments when using cultured cardiomyocytes. They are presented as mean ± SEM. The comparison between the experimental groups was performed using ANOVA and two-tailed Student's *t*-test for unpaired samples with normality test and Bonferroni correction when analyzing simple time points (GraphPad Instat.V3.05). The optimal number of experiments per time point and group was chosen in accordance with our previous experience [11]. The level of statistical significance was set at $p < 0.05$.

3. Results

3.1. rhEpo and I/R myocardial damage

Significant reduction of the cardiac TnT plasma levels in the rhEpo-treated group indicated that intravenous application of the rhEpo could efficiently reduce both early and late reperfusion-induced myocardial injury (Fig. 1). In particular, rhEpo abolished acute release of the atrial and brain natriuretic factors into the circulation upon restoration of blood perfusion of the graft (Fig. 2). Systemic inflammatory response assessed as an increase in plasma IL-6 level 6 h after the onset of perfusion was not affected by rhEpo treatment (235 ± 72 pg/ml vs 291 ± 67 pg/ml in control and rhEpo-treated recipients, $p > 0.05$). Thus, cardioprotective effect of Epo was not due to the suppression of the secondary inflammatory response.

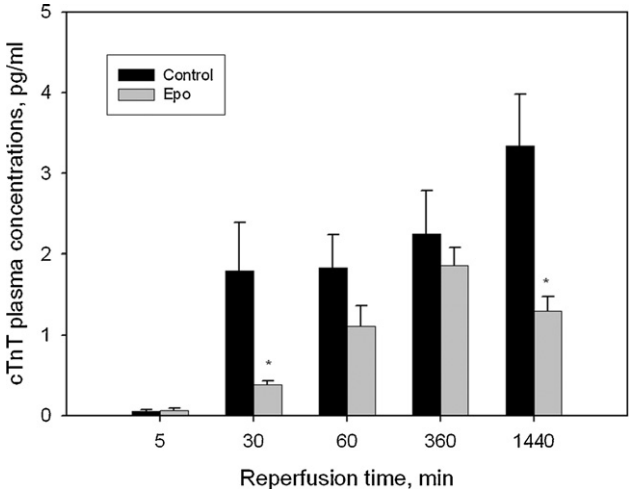


Fig. 1. Plasma troponin T levels. Data are means of 6–8 independent experiments ± SEM (n = 6–8). * indicates $p < 0.05$ compared to the corresponding time point in non-treated control group.

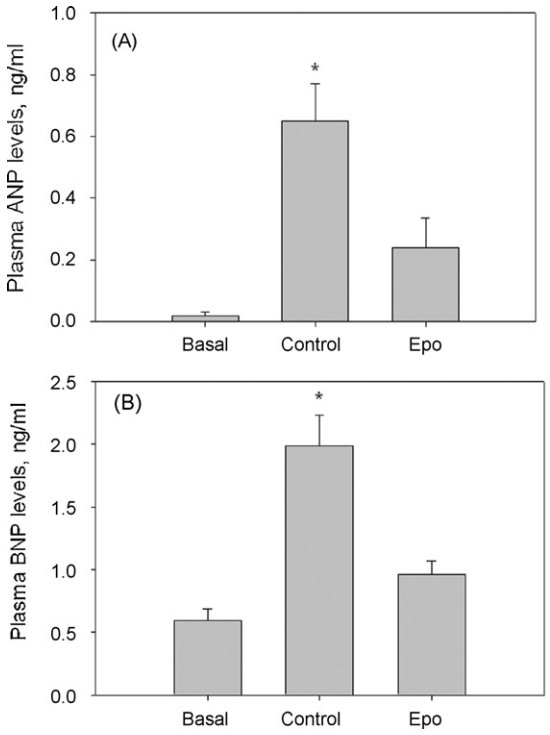


Fig. 2. (A) Plasma levels of atrial natriuretic peptide ANP in non-treated control animals (basal) and 5 min after the onset of reperfusion in non-treated control animals (control) and rhEpo-treated animals (Epo). Data are means of 6–8 independent experiments ± SEM. * indicates $p < 0.05$ compared to the corresponding time point in non-treated control group. (B) Plasma levels of brain natriuretic peptide BNP in non-treated control animals (basal) and 5 min after the onset of reperfusion in non-treated control animals (control) and rhEpo-treated animals (Epo). Data are means of 6–8 independent experiments ± SEM. * indicates $p < 0.05$ compared to the corresponding time point in non-treated control group.

The time from the onset of reperfusion to the defibrillation of the grafted heart was shorter in rhEpo-treated than non-treated group (47 ± 2 s vs 66 ± 4 s, respectively, n = 40, p = 00012).

3.2. Mechanisms of I/R injury

Interaction of rhEpo with its receptor was shown to reduce apoptosis in isolated cardiomyocytes [3]. We have evaluated the degree of apoptosis in transplanted hearts of control and rhEpo-treated animals as well as between the transplanted and native hearts.

To do so we have assessed caspase 9 and caspase 3 activity in ventricular tissue homogenate. The resulting activity was compared between native and transplanted hearts in both control and Epo-treated animal groups and related to the activity of caspases in cell culture where apoptosis was triggered by Staurosporine treatment. The basal activity of caspase 3 and caspase 9 in the native

Please cite this article in press as: Mihov D, et al. Erythropoietin protects from reperfusion-induced myocardial injury by enhancing coronary endothelial nitric oxide production. Eur J Cardiothorac Surg (2009), doi:10.1016/j.ejcts.2008.12.049

heart tissue was very low (0.28% of the positive control, Staurosporine-treated vascular endothelial cells for caspase 3 and 0.20% of the positive control for caspase 9). Ischemia–reperfusion resulted in a very modest increase of the activity of both caspases which was only statistically significant between the native and the transplanted hearts 30 min after the onset of perfusion (data not shown). This difference was insignificant when caspases' activity in transplanted hearts of Epo-treated group were compared to the native heart levels. Activation of caspase 3 did not cause detectible PARP cleavage in transplanted hearts at any reperfusion time point. In addition, the number of TUNEL-positive cells ranged between 0 and 4 cells/×20 field and did not differ statistically between non-treated and rhEpo-treated ischemic hearts. Taken together with the data on cardiac TnT release into the circulation these data suggest that most of the ischemia–reperfusion damage represents oncosis rather than apoptosis.

3.3. Cellular ion and water content

Data on tissue water and sodium content shown in Fig. 3A and B provide further confirmation for the preferential oncotic acute reperfusion damage. Reperfusion is followed by a transient increase in tissue sodium and water levels in transplanted hearts of control but not in rhEpo-treated animals. Recovery of the tissue ion/water balance occurred within 30 min of reperfusion in both control grafts and rhEpo-treated grafts.

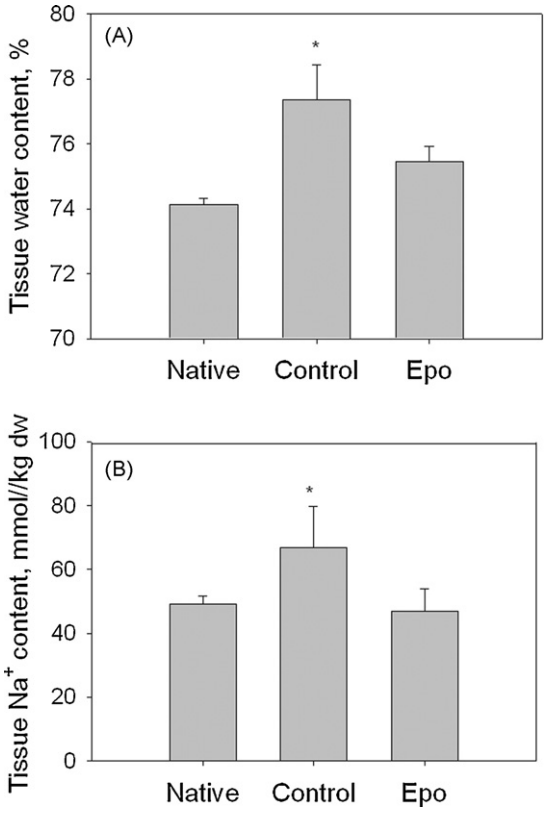


Fig. 3. Effect of I/R and rhEpo on the tissue water and Na^+ content. (A) Water content in ventricular tissue of native non-ischemic hearts of the non-treated control group (native) and in ischemic reperfused hearts of non-treated control group (control) and rhEpo-treated group (Epo) 5 min after the onset of perfusion. (B) Tissue Na^+ content in the same set of samples. Data are means of 6–8 hearts ± SEM. * indicates $p < 0.05$ compared to the value in native hearts.

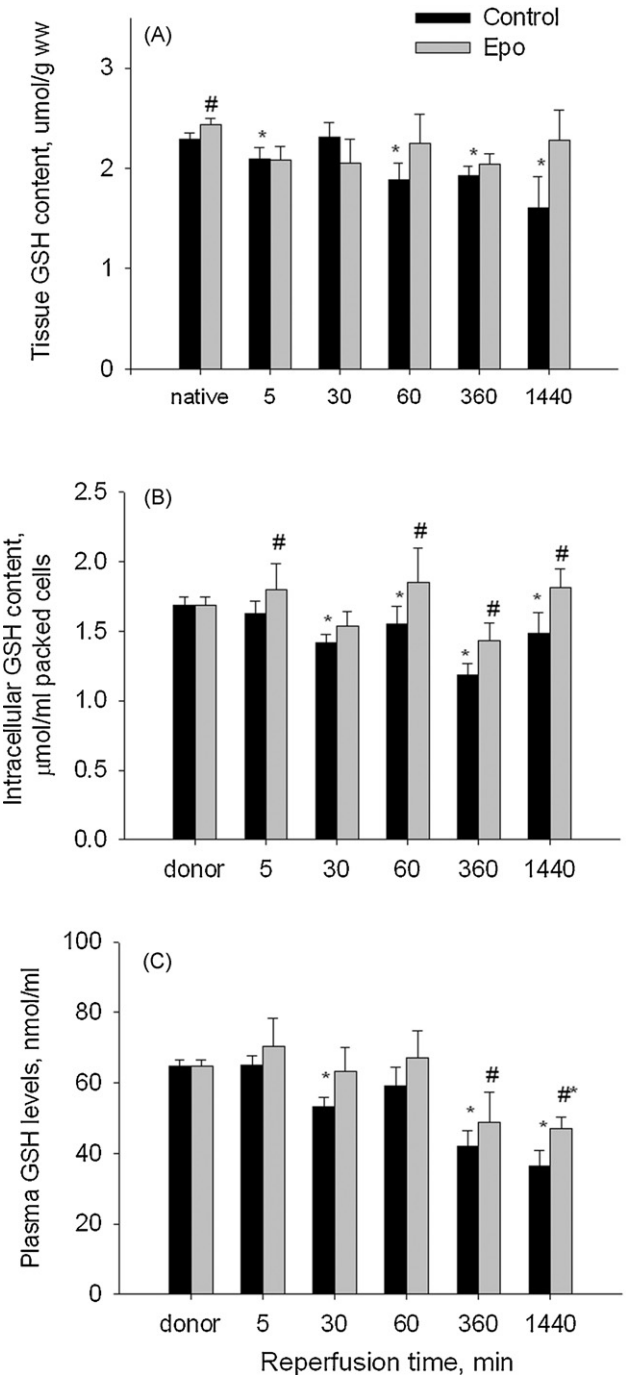


Fig. 4. Reduced glutathione levels in ventricular tissue homogenates of native non-treated (n.control) and native rhEpo-treated (n.Epo) hearts, erythrocytes and plasma. (A) GSH pool of ventricular tissue. Values are means of 6–8 independent heart samples ± SEM. Note that the values for native hearts differ between the control and rhEpo-treated animals (denoted with #, $p < 0.05$). * indicates $p < 0.05$ compared to the native control value. (B) and (C) GSH levels in erythrocytes and plasma. Data are means of 6–8 experiments ± SEM. # indicates $p < 0.05$ compared to the levels in non-treated control animals of the corresponding time point. * denotes $p < 0.05$ compared to the basal level in non-treated animals.

Please cite this article in press as: Mihov D, et al. Erythropoietin protects from reperfusion-induced myocardial injury by enhancing coronary endothelial nitric oxide production. Eur J Cardiothorac Surg (2009), doi:10.1016/j.ejcts.2008.12.049

3.4. Reperfusion and oxidative stress

Oxidation is one of the recognized causes of the myocardial damage at reperfusion. Indeed, reperfusion resulted in acute depletion of the GSH pool in ventricular tissue that became even more pronounced with time (Fig. 4A). Administration of rhEpo resulted in an increase in the GSH content in native myocardium ($p < 0.05$). Transplanted hearts of the rhEpo-treated group did not show GSH depletion at any reperfusion time point (Fig. 4A). Intravenous administration of rhEpo also abolished reduction of the GSH levels in erythrocytes and plasma of the recipient animals thus providing systemic defense from the reperfusion-induced oxidative stress (Fig. 4B and C).

3.5. Mechanisms of the cardioprotective action of rhEpo

As shown in Figs. 3 and 4, rhEpo protects transplanted myocardium from reperfusion-induced oxidative stress, edema and Na^+ accumulation. Plasma nitrite levels in recipient animals were significantly upregulated following rhEpo administration indicating an increase in nitric oxide production (Fig. 5). The source of NO production was characterized by using antibodies against phosphorylated (active) forms of endothelial nitric oxide synthase. Interestingly, rhEpo treatment resulted in activation of eNOS selectively in vascular endothelium of coronary vessels but not in the myocardial tissue itself (Fig. 6C and D). Cardiac myocytes are known to express eNOS along with vascular endothelial cells. We therefore investigated localization of rhEpo in both native and transplanted heart tissue. The data presented in Fig. 6A and B indicate that rhEpo does not diffuse from the lumen of the coronary vessels into the myocardium at least for the first 30 min of reperfusion. Both the rhEpo binding and activation of the eNOS are restricted to the endothelial compartment.

In order to address the possible effect of rhEpo on ion and water equilibrium in more details we have monitored the active and passive K^+ transport across the sarcolemmal membrane of primary cultures of cardiac myocytes in the

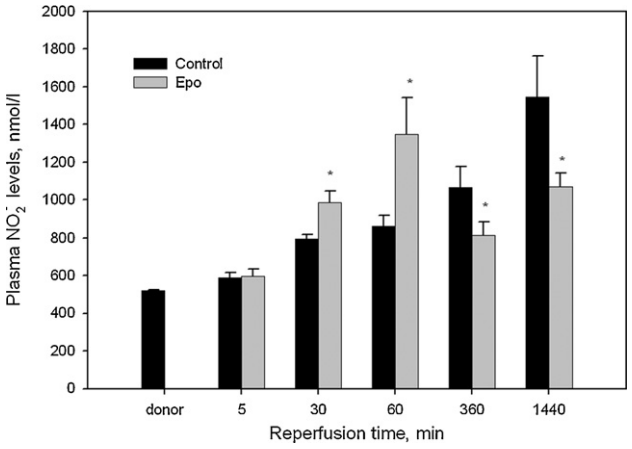


Fig. 5. Nitrite levels in plasma of rhEpo-treated animals (rhEpo) and non-treated control animals (control). Data are means of 6–8 animals ± SEM. * denotes $p < 0.05$ compared to the corresponding time point in non-treated control animals.

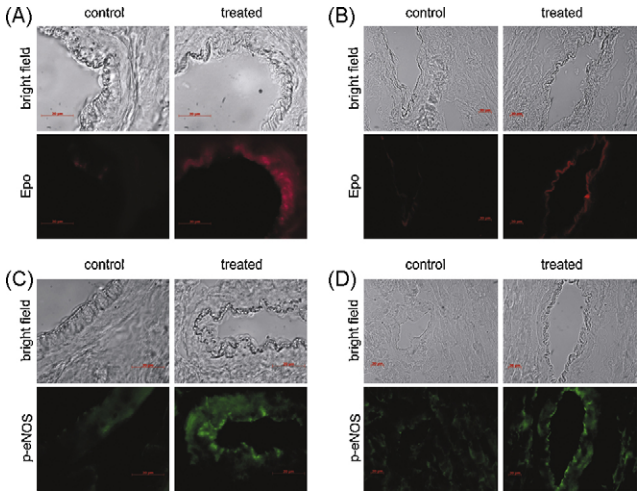


Fig. 6. Localization of the rhEpo binding (stained in red) and eNOS activation (phosphorylated active form stained in green) in the grafts. rhEpo localization in the ventricular tissue of the grafts 5 min after the onset of reperfusion (A) and 30 min after the onset of perfusion (B). Localization of the phospho-eNOS (antibodies against phosphor-Ser 1177) in the transplanted hearts 5 min (C) and 30 min (D) after the onset of perfusion. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

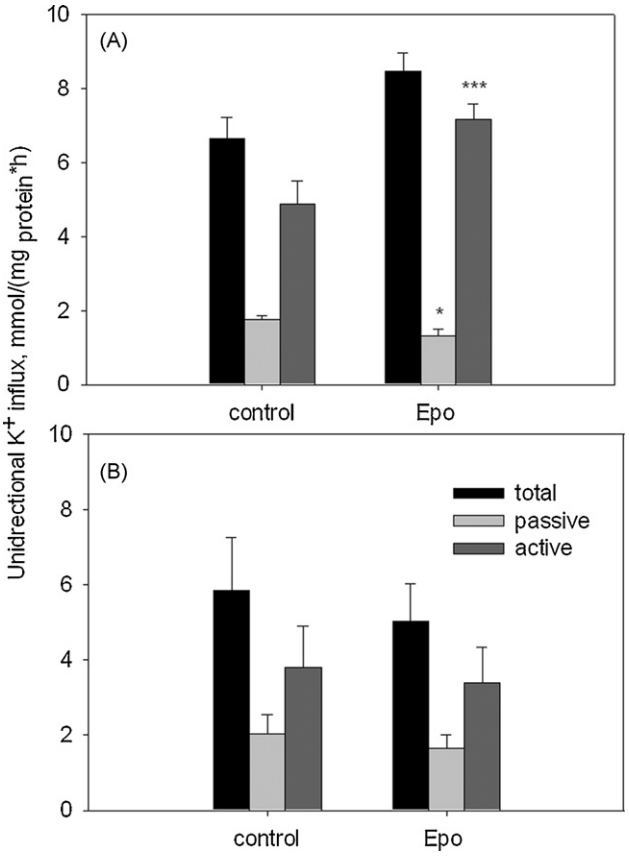


Fig. 7. Total, passive and active K^+ influx components into neonatal rat cardiomyocytes in the presence or in the absence of 10 U/ml rhEpo in the medium containing 100 μM L-arginine (A) or in arginine-free medium (B). Data are means of five independent experiments ± SEM. *** and * indicates $p < 0.001$ and $p < 0.05$ correspondingly when compared to the Epo-free conditions.

Please cite this article in press as: Mihov D, et al. Erythropoietin protects from reperfusion-induced myocardial injury by enhancing coronary endothelial nitric oxide production. Eur J Cardiothorac Surg (2009), doi:10.1016/j.ejcts.2008.12.049

presence and in the absence of rhEpo. Administration of rhEpo resulted in stimulation of the active influx of K^+ mediated by the Na/K ATPase along with suppression of passive K^+ movement across the sarcolemma (Fig. 7A). This does not occur when the cell incubation medium is deprived of L-arginine, a substrate of eNOS (Fig. 7B) suggesting the observed effects of rhEpo on both the Na/K ATPase and the passive K^+ flux are not direct but secondary to the Epo-induced stimulation of NO production.

4. Discussion

Choosing an in vivo model of cold global ischemia and warm reperfusion we intended to mimic as closely as possible the clinical setting during open-heart surgical procedures. The narrow time window of cardioprotection for rhEpo makes it almost useless when treating myocardial infarction (first hours after the injury) [3,12]. The preoperative period on the contrary allows precisely timed administration of the drug assuring maximal cardioprotective effect of rhEpo. To our knowledge, the present study is the first one investigating the myocardial action of rhEpo during cold global I/R injury in vivo. Our data indicate that in i.v. administration of rhEpo after the onset of cold global ischemia but prior to warm reperfusion confers an acute cardioprotective effect in rats. This cardioprotection offered by rhEpo was not linked to the suppression of apoptosis but to the reduction of oxidative stress and edema, which was at least partially mediated by the rhEpo-induced stimulation of NO production.

This observation is in line with numerous reports of cardioprotective efficiency of rhEpo obtained using different models and experimental settings including isolated rat cardiomyocytes [6,13–15], in vitro Langendorff model [6,13,16–18] and in vivo coronary artery ligation model [12,14,15,19]. Despite extensive investigations the mechanisms of rhEpo-induced cardioprotection remain a matter of debate. Data generated using primarily isolated cardiomyocytes, but also in some in vivo models suggest that rhEpo protects myocardium from apoptosis [12,15]. However, the role of apoptosis of cardiomyocytes in I/R injury is questionable [1,20]. Our data reveal that, although occurring, apoptosis does not contribute significantly to the cold global I/R injury in our experimental model. Reperfusion induced only marginal activation of caspase 9 and caspase 3 at only one time point (30 min of reperfusion). However, at 30 min of reperfusion rhEpo administration significantly reduced myocardial cell injury reflected by plasma troponin (Fig. 1). Of note, reduction in mechanical overload evidenced by ANP and BNP release into the plasma was detectible even earlier, already 5 min after the onset of blood flow (Fig. 2). We were unable to monitor the changes in downstream targets of caspase activation such as PARP cleavage or DNA fragmentation suggesting that when occurring, apoptotic response was incomplete. Very close to our results, van der Meer and co-workers found only a modest proportion of apoptotic cells positive for active caspase 3 in rat hearts subjected to ischemia–reperfusion with ($1.8 \pm 0.09\%$) or without ($2.1 \pm 0.12\%$) rhEpo treatment [18]. Interestingly, activation of the caspase 3 in their study was mainly restricted to the endothelial cells and fibroblasts. This finding is further supported by Scarabelli and co-workers reporting that apoptosis of endothelial cells precedes cardiomyocyte cell apoptosis in ischemia/reperfusion injury [20]. Parsa and co-workers suggested a potential role of cardiac fibroblasts in modulating cardioprotective effects of Epo in hearts subjected to ischemia–reperfusion [14].

Edema and Na^+ accumulation in the myocardial tissue we have observed (Fig. 3) as well as delayed inflammatory response are also not consistent with the concept of apoptosis as a dominating course of I/R injury. Histological examination revealed extensive myocardial lesions where cell membranes lacked integrity, which became detectible just 30 min after the onset of perfusion when cardiac TnTwas detected in plasma of recipient animals (Fig. 1). Taken together these findings suggest that most of the myocardial damage represented oncotic necrosis.

Our data furthermore indicate that intravenous rhEpo administration would fail to suppress apoptosis in the myocardium even if the latter were occurring for one single reason: rhEpo does not cross the endothelial barrier and hence does not reach the myocardium at least during the first hours of reperfusion (Fig. 6). Thus, data obtained for interaction of the cytokine with isolated cardiomyocytes [15] cannot be compared with those obtained using ex vivo or in vivo models. The target for rhEpo applied intravenously is primarily the endothelium of coronary vessels. Upon binding to the endothelial cells occurring instantaneously (Fig. 6) rhEpo causes activation of the NO production (Fig. 5) and most likely other factors such as endothelin 1 [17] that are mediating cardioprotective effects observed by us and others.

Among the final targets of the rhEpo are reduction of mechanical overload monitored as release of the stress factors ANP and BNP, abrogation of the reperfusion-induced oxidative stress (Fig. 4) and accelerated recovery of the ion and water balance in the myocardial tissue (Fig. 3). It is unlikely that the observed multiple effects are caused by activation of a single signaling pathway. Detailed investigation of involved molecular events in potential signaling pathways is far beyond the scope of this study. Similar suppression of the ANP and BNP release upon reperfusion following ischemia in rhEpo-treated animal models was reported by others [17,19]. Antioxidative effect of rhEpo was also shown in different tissues and experimental settings [3].

We suggest that activation of the NO production in the endothelial cells is at least in part responsible for the lack of GSH depletion in myocardial tissue during reperfusion. There is long-standing debate as to whether NO plays a beneficial or detrimental role in ischemia–reperfusion injury. It seems tempting to extrapolate the results of in vitro experiments using cell cultures to the in vivo pathogenic conditions [21]. Redox environment is the factor decisive for both NO bioavailability and the end products formed which may include both nitrite, nitrate, S- and N-nitrosylation adducts and nitrotyrosine. It has been shown that lower levels of NO production and diffusion into the tissue are associated with its antioxidative properties [22]. The redox environment can in turn modulate the NO function and potentiate its cardioprotective action. This multifaceted action of NO defines its narrow therapeutic safety window for NO in ischemia–reperfusion pathophysiology [22]. In the present

Please cite this article in press as: Mihov D, et al. Erythropoietin protects from reperfusion-induced myocardial injury by enhancing coronary endothelial nitric oxide production. Eur J Cardiothorac Surg (2009), doi:10.1016/j.ejcts.2008.12.049

study, administration of rhEpo was followed by increased phosphorylation and activation of eNOS with subsequent enhanced production of NO during the early phase of reperfusion. Upregulation of NO production was associated with reduction of oxidative stress and consequently of myocardial injury. This is in accordance with previous report of Bullard and co-workers who showed that the cardioprotective effect of Epo was associated with a two-fold increase in phosphorylated eNOS [13].

Along with its role as a scavenger of superoxide anions NO has been shown to modulate numerous processes including ion transport [23]. In our study, reduction in myocardial injury was also accompanied by a decrease in tissue edema and Na^+ accumulation. We demonstrated that activation of the NO production triggered by rhEpo treatment of neonatal rat cardiomyocytes (our observations as well as [13,17,21]) modulated Na/K ATPase activity. This is the first report on the role of NOS in regulation of the active K^+ transport in cardiomyocytes. However, our recent study revealed similar coupling between NOS-mediated NO production and activity of the Na/K ATPase in cerebellar granule cells [23]. Along with activation of the Na/K ATPase reduction of the passive K^+ fluxes is observed in the cardiomyocytes where NO generation is induced by rhEpo furthermore facilitating restoration of the transmembrane ion gradients (Fig. 7).

There are limitations in the present study related to its design. Focusing mainly on investigating the mechanisms of Epo-induced cardioprotection, we chose a fixed dose at a fixed time of administration of rhEpo. However, the dose of 5000 U/kg rhEpo used in our study has been shown to confer significant cardioprotection against ischemia–reperfusion [12]. After demonstrating the cardioprotection conferred by rhEpo, we limited our study to the potential role of NO alone. We cannot exclude that other local or systemic factors modulated by rhEpo such as endothelin 1, vascular endothelial growth factor and transforming growth factor may play a significant role in the acute and late cardioprotection induced by rhEpo [3,17]. The involvement of other secondary effectors in the cardioprotection offered by rhEpo warrants further investigations. Moreover, our rodent model does not allow to assess clinical relevance of the obtained results but gives insight for further characterization of mechanisms of potential cardioprotection offered by Epo in human settings.

In summary, intravenous administration of rhEpo after the onset of ischemia but prior to reperfusion protects the heart against cold global I/R. Apoptosis does not seem to play a major role in the process of tissue injury in this model. After binding to the coronary endothelium, rhEpo enhances NO production by phosphorylation and thus activation of endothelial nitric oxide synthase in coronary vessels. Our data suggest that cardioprotective properties of rhEpo are at least partially mediated by nitric oxide released by the coronary endothelium.

Acknowledgment

The authors acknowledge the excellent technical assistance of Mrs Vlasta Strohmeier.

References

[1] Murphy E, Steenbergen C. Mechanisms underlying acute protection from cardiac ischemia–reperfusion injury. Physiol Rev 2008;88:581–609.
[2] Bolli R, Becker L, Gross G, Mentzer Jr RM, Balshaw D, Lathrop DA. Myocardial protection at a crossroads. The need for translation into clinical therapy. Circ Res 2004;95:125–34.
[3] Arcasoy MO. The non-haematopoietic biological effects of erythropoietin. Br J Haematol 2008;141:14–31.
[4] Junk AK, Mammis A, Saviz SI, Singh M, Roth S, Malhotra S, Rosenbaum PS, Cerami A, Brines M, Rosenbaum DM. Erythropoietin administration protects retinal neurons from acute ischemia–reperfusion injury. Proc Natl Acad Sci U S A 2002;99:10659–64.
[5] Wang Y, Zhang ZG, Rhodes K, Renzi M, Zhang RL, Kapke A, Lu M, Pool C, Heavner G, Chopp M. Post-ischemic treatment with erythropoietin or carbamylated erythropoietin reduces infarction and improves neurological outcome in a rat model of focal cerebral ischemia. Br J Pharmacol 2007;151:1377–84.
[6] Wright GL, Hanlon P, Amin K, Steenbergen C, Murphy E, Arcasoy MO. Erythropoietin receptor expression in adult rat cardiomyocytes is associated with an acute cardioprotective effect for recombinant erythropoietin during ischemia–reperfusion injury. FASEB J 2004;18:1031–3.
[7] Ono K, Lindsey ES. Improved technique of heart transplantation in rats. J Thorac Cardiovasc Surg 1969;57:225–9.
[8] Bombeli T, Schwartz BR, Harlan JM. Endothelial cells undergoing apoptosis become proadhesive for nonactivated platelets. Blood 1999;93:3831–8.
[9] Tietze F. Enzymic method for quantitative determination of nanogram amounts of total and oxidized glutathione: applications to mammalian blood and other tissues. Anal Biochem 1969;27:502–22.
[10] Schafer BW, Perriard JC. Intracellular targeting of isoproteins in muscle cytoarchitecture. J Cell Biol 1988;106:1161–70.
[11] Tavakoli R, Gazdhar A, Pierog J, Bogdanova A, Gugger M, Pringle IA, Gill DR, Hyde SC, Genoni M, Schmid RA. Electroporation-mediated interleukin-10 overexpression in skeletal muscle reduces acute rejection in rat cardiac allografts. J Gene Med 2006;8:242–8.
[12] Moon C, Krawczyk M, Paik D, Lakatta EG, Talan MI. Cardioprotection by recombinant human erythropoietin following acute experimental myocardial infarction: dose response and therapeutic window. Cardiovasc Drugs Ther 2005;19:243–50.
[13] Bullard AJ, Govewalla P, Yellow DM. Erythropoietin protects the myocardium against reperfusion injury in vitro an in vivo. Basic Res Cardiol 2005;100:397–403.
[14] Parsa CJ, Kim J, Riel RU, Pascal LS, Walton GB, Thompson RB, Petrofski JA, Matsumoto A, Stamler J, Koch WJ. Cardioprotective effects of erythropoietin in the reperfused ischemic heart. A potential role for cardiac fibroblasts. J Biol Chem 2004;279:20655–62.
[15] Tramontano AF, Muniyappa R, Black AD, Blendea MC, Cohen I, Deng L, Sowers JR, Cutaia MV, El-Sherif N. Erythropoietin protects cardiac myocytes from hypoxia-induced apoptosis through an Akt-dependent pathway. Biochem Biophys Res Commun 2003;308:990–4.
[16] Hanlon PR, Fu P, Wright GL, Steenbergen C, Arcasoy MO, Murphy E. Mechanisms of erythropoietin-mediated cardioprotection during ischemia–reperfusion injury: role of protein kinase C and phosphatidylinositol-3-kinase signalling. FASEB J 2005;19:1323–5.
[17] Piuhola J, Kerkela R, Keenan JI, Hampton MB, Richards AM, Pemberton CJ. Direct cardiac actions of erythropoietin (EPO): effects on cardiac contractility. BNP secretion and ischaemia/reperfusion injury. Clin Sci (Lond) 2008;114:293–304.
[18] van der Meer P, Lipsic E, Henning RH, de Boer RA, Suurmeijer AJH, van Veldhuisen DJ, van Gilst WH. Erythropoietin improves left ventricular function and coronary flow in an experimental model of ischemia–reperfusion injury. Eur J Heart Fail 2004;6:853–9.
[19] van der Meer P, Lipsic E, Henning RH, Boddeus K, van der Velden J, Voors AA, van Veldhuisen DJ, van Gilst WH, Schoemaker RG. Erythropoietin induces neovascularization and improves cardiac function in rats with heart failure after myocardial infarction. J Am Coll Cardiol 2005;46:125–33.
[20] Scarabelli T, Stephanou A, Rayment N, Pasini E, Comini L, Curello S, Ferrari R, Knight R, Latchman D. Apoptosis of endothelial cells precedes myocyte cell apoptosis in ischemia/reperfusion injury. Circulation 2001;104:253–6.
[21] Burger D, Lei M, Geoghegan-Morphet N, Lu X, Xenocostas A, Feng Q. Erythropoietin protects cardiomyocytes from apoptosis via up-regulation of endothelial nitric oxide synthase. Cardiovasc Res 2006;72:51–9.

Please cite this article in press as: Mihov D, et al. Erythropoietin protects from reperfusion-induced myocardial injury by enhancing coronary endothelial nitric oxide production. Eur J Cardiothorac Surg (2009), doi:10.1016/j.ejcts.2008.12.049

[22] Pacher P, Beckman JS, Liaudet L. Nitric oxide and peroxynitrite in health and disease. Physiol Rev 2007;87:315–424.
[23] Petrushanko IY, Bogdanov NB, Lapina N, Boldyrev AA, Gassmann M, Bogdanova AY. Oxygen-induced regulation of Na/K ATPase in cerebellar granule cells. J Gen Physiol 2007;130:389–98.

Appendix A. Conference discussion

Dr T. Wahlers (*Cologne, Germany*): Yesterday in the Young Investigators Award, the action of Epo on stem cells was reported and they used in rats a dose of 3000 units/kg. You used 5000. Can you give us your rationale using this high dose of Epo and transfer this perhaps to the human side.

Dr Tavakoli: I think one should make a difference between the cell culture model or in vivo model. Besides, the aim of our study was just to prove the cardioprotective potential of Epo and, as I mentioned, get insights into its mechanism. We chose this dose and also the time of administration based on previous studies, again, in an in vivo regional model where the authors have shown that 5000 units/kg is efficient [ref. 19 in the manuscript]. I think in the acute setting of this study the proliferative properties of Epo wouldn't be a major problem.

Dr J. Vaage (*Oslo, Norway*): I think you have a very interesting model by transplanting the hearts back to the animals. I have two questions for you.

You showed that the erythropoietin binds to the endothelium, but you gave it only very briefly before taking out the heart. I think it was 20 min.

Dr Tavakoli: Right.

Dr Vaage: Do you have any data or any information that if you keep it, let's say, 6 h or 24 h, before you could find it located in other places like, for instance, the cardiomyocytes. That's my first question.

My second is more comment. You claim that the effect is partly mediated by endothelial NO. I actually refute that, because you just showed that it is upregulated, which is not in any way an indication that it is the mediator. In order to show that you need either pharmacological blocking or using mice with knockout NO.

Dr Tavakoli: First, your first question about the timing of the application, as I mentioned, this was not a study to investigate the optimal timing of Epo injection. The curve I've shown you indicates that the half-life of Epo is about 2 h. This information was obtained after we started the study, so we did not change the timing and the doses of Epo administration.

Regarding your second input, you know that NO itself is an antioxidative molecule, so this is the reason I suggest that NO is related to the antioxidative and cardioprotective effect of Epo in this model. Therefore, it's one explanation for my assertion that NO is involved in this cardioprotection and antioxidative action of Epo.

Dr Vaage: If I may comment on it. There is, let's say, scientific background for making that suggestion, but I think we must make it very clear at this stage of your investigation it's only a suggestion.

Dr Tavakoli: Correct. It's a suggestion. To be able to say our data indicate that NO is related, we should have put an inhibitor of eNOS and shown that it could abolish the effect of EPO. Hence, it warrants further investigation. I think the best way to say it indicates is that you block the effect of EPO with inhibitors of eNOS.

Please cite this article in press as: Mihov D, et al. Erythropoietin protects from reperfusion-induced myocardial injury by enhancing coronary endothelial nitric oxide production. Eur J Cardiothorac Surg (2009), doi:10.1016/j.ejcts.2008.12.049

9. CURRICULUM VITAE

- **MIHOV**
- **Deyan Nikolaev**
- **26.01.1979**
- **Bulgarian**

- **Education:**

1992 - 1997 - Foreign Language School (Gymnasium), Varna, Bulgaria

- profile: French and Russian

1997- 2002 - Sofia University, Bulgaria

2002 - Master of Science in Molecular Biology

- diploma subject: **Vascular Physiology**
- title of diploma thesis - **Role of heme oxygenase in regulation of spontaneous contraction activity of guinea-pig portal vein**

2005 – 2009 - PhD student, University of Zurich

- Institute of Veterinary Physiology and Zurich Center of Integrative Human Physiology (ZIHP), University of Zurich

- **Publications and manuscripts during doctorate studies**

Publications:

1) ***Mihov D***, Bogdanov N, Grenacher B, Gassmann M, Zund G, Bogdanova A, Tavakoli R. Erythropoietin protects from reperfusion-induced myocardial injury by enhancing coronary endothelial nitric oxide production. Eur J Cardiothorac Surg (2009), doi:10.1016/j.ejcts.2008.12.049
2) Dimitrova D, ***Mihov D***, Wang R, Hristov K, Rizov L, Bolton T, Duridanova D. Contractile effect of ghrelin on isolated guinea-pig renal arteries. Vascul Pharmacol. 2007 Jul;47(1):31-40. Epub 2007 Apr 5.
3) Bogdanova A, ***Mihov D***, Lutz H, Saam B, Gassmann M, Vogel J. Enhanced erythro-phagocytosis in polycythemic mice overexpressing erythropoietin. Blood. 2007 Mar 29
4) ***Mihov D***, Duridanova D. Ghrelin-induced contraction of guinea-pig renal artery. COMPTES RENDUS DE L'ACADEMIE BULGARE DES SCIENCES. 2005; V 58 (6); 717-722

Submitted manuscripts:

- ***Mihov D***, Vogel J, Gassmann M, Bogdanova A. Erythropoietin activates nitric oxide synthase in murine erythrocytes. submitted in *American Journal of Physiology, C-00543-2008*, currently under revision

Manuscripts in preparation:

- Bogdanova A, ***Mihov D***, Grenacher B, Vogel J, Gassmann M, Tavakoli R. Erythropoietin binding in the heart in vivo and in vitro: behind cardioprotection

• Participations in congresses

Oral presentations:

- 7th International Lübeck Conference on the pathophysiology and pharmacology of erythropoietin and other hematopoietic growth factors. Lübeck, Germany, September, 2006

Poster presentations:

1) annual USGEB meeting, Interlaken, Switzerland, January – 2009
2) 4th ZIHP symposium, Zurich, Switzerland, August – 2008 – **awarded with the best poster prize**
3) Hypoxia, from Integrative Biology to Human Disease Monte Verità, Ascona, Switzerland, November - 2007
4) Annual Meeting of the Swiss Physiological Society, Bern, Switzerland, September – 2007
5) 3rd ZHIP symposium, Zurich, Switzerland, August – 2007
6) 2nd ZHIP symposium, Zurich, Switzerland, August - 2006
7) annual USGEB meeting, Geneva, Switzerland, February – 2006

10. ACKNOWLEDGEMENTS

First of all, I would like to thank to Prof. Max Gassmann, who gave me the great opportunity to become part of his lab and to do my PhD work in the exciting field of hypoxia and erythropoietin physiology.

I would like to express my special gratitude to my supervisor Dr. Anna Bogdanova, who is outstanding scientist, great person and a good friend. I am extremely grateful for her patience, trust and guidance throughout these four years.

I have special thanks to Dr. Hans Lutz and Prof. Christian Grimm for their tutorship and counsels as a members of my PhD thesis committee.

I would like to give my appreciation to Dr. Reza Tavakoli and Vlasta Strohmeier for their fruitful collaboration. I am particularly grateful to Prof. Johannes Vogel for his strong scientific partnership and guidance.

Special thanks go to Mikola Bogdanov for his crucial help and assistance during my first days in the lab. His technical support, jokes and great sense of humor make the life behind the bench much nicer and easier.

Sincere thanks to the former and current rulers of the lab – Beat Grenacher, Bianca Saam Stephan Keller and Michaela Bednar for the technical assistance and support.

Also I would like to express my thanks to Dr. Lara Ogunshola, Abraham Al Ahmad and Carole Bürgi-Taboada for shearing with me their scientific knowledge, language- and computer skills.

Many thanks to all the members of Gassmann's group including Dr. Thomas Gorr, Dr. Jorge Soliz, Dr. Beat Schuler and my PhD fellows Milena Komniski, Sergey Yakushev, Asya Makhro, Junmin Hu, Ivan Hang, Zhenghui Wang for the great scientific and friendly atmosphere in the lab.

I would like to express my special gratitude to my wife Maria Asparuhova, which always supports me in good and bad times. I want to thank her for all the patience, understanding and love she is constantly giving to me. In my PhD life of devoted Epo-physiologist, Maria's way of thinking and advices as a molecular biologist were priceless.

Finally I would like to thank to all my family, especially to my parents - Danilina and Nikolai and Maria's parents – Vania and Bozhidar, for their constant encourage and support.